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14. ABSTRACT

The most common mode of HIV transmission is through sexual contact, and as such semen is the vector that is fueling the worldwide spread of the virus. We and our colleagues have identified and characterized amyloid fibrils from human semen that substantially promote HIV infection. In our funded proposal, we describe experiments to study the components of semen that enhance HIV infection, to decipher their mechanisms of action both *in vitro* and *in vivo*, and to identify methods of blocking their activity. We have made significant progress in our first year of the funded proposal. In addition to identifying a novel set of viral enhancing factors from semen, we have identified new mechanisms by which these amyloids can promote HIV transmission. We have shown an association between the levels of semen amyloids with endogenous viral load in samples form HIV-infected men, and demonstrated the activity of the amyloids *in vivo* in a humanized mouse model of HIV transmission. Finally, we have initiated a small-molecule screen for inhibitors of these amyloids, and have identified promising hits that we are currently further testing in secondary analysis. Inhibiting the activity of these semen factors can lead to the development of a new generation of microbicides targeting HIV together with naturally-occurring viral enhancement factors.

15. SUBJECT TERMS
HIV, AIDS, Transmission, Semen, SEVI (Semen-derived Enhancer of Viral Infection), SEM (Semenogelin), Amyloid fibrils, Animal model, Infection, Inhibitors, Inflammation

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Introduction

The goals of our proposal entitled “A Novel Approach for Preventing HIV Infection and Reducing Risk to US Military Personnel” are to understand the mechanisms by which semen enhances HIV infection and to identify ways to block this activity. Far from being a passive vehicle for HIV during sexual transmission, semen and seminal fluid greatly enhance viral infectivity when tested under conditions that minimize or preclude its long-known cytotoxic effects (1-11). In 2007, our colleagues identified Semen-derived Enhancer of Viral Infection [SEVI], semen amyloids that greatly augment HIV infectivity (3). We subsequently deciphered one mechanism by which SEVI promotes HIV infection (5) and identified a small molecule inhibitor of its activity (6). In this proposal, we set out to determine whether other factors in semen promote HIV infection, and if so to identify their mechanisms of action. We further study the effects of semen amyloids on HIV transmission *in vivo*, and set up a screen to identify compounds that can inhibit the ability of semen to enhance HIV infection. Safe and effective inhibitors of semen-derived viral enhancers may prove useful as components of anti-HIV microbicides.

BODY

We describe in this section the research accomplishments associated with the five aims outlined in the approved Statement of Work [SOW].

Aim 3

In this aim, we proposed to identify new factors in semen that promote HIV infection. We start with progress on this aim, because it is here that we have made the most progress over the past year.

Identification of HIV-enhancing SEM amyloids from human semen

In sub aim 3.2, we proposed to use the amyloid-specific antibody W01 to identify new amyloid fibrils from human semen. We found that W01 specifically bound fragments from the N-terminus of semenogelin [SEM], an abundant semen protein that is the major constituent of the semen coagulum. We confirmed that these peptides form amyloid fibrils that efficiently enhance HIV infection. Furthermore, semen samples naturally deficient in these proteins lack viral enhancement activity. The progress made in this sub aim are detailed in a manuscript that has been published in the journal *Cell Host and Microbe* (9). For reference, we have attached a copy of the manuscript, wherein the DOD is acknowledged as a funding source. The identification of SEM fibrils suggests that there are at least two abundant proteins from human semen that harbor amyloidogenic peptides: PAP, the precursor to the SEVI amyloids previously identified (3), and SEM (9). Much of the remaining Annual Report focuses on the SEM fibrils and not SEVI, because the precursor for the SEM fibrils is more abundant than the precursor for SEVI, and as such SEM fibrils may play a bigger role in the viral enhancement activity of semen. However, we note that the biophysical properties of SEVI and SEM fibrils are highly similar (9).

Characterization of the role of semen fibronectin in enhancing HIV infection

In sub aim 3.1, we proposed to fractionate human seminal fluid, and then analyze fractions by mass spectrometry (MS) iTRAQ coupled to HIV infectivity assays. We found multiple fractions that enhanced HIV infection, one of which contained seminal fibronectin (as identified by MS iTRAQ). As fibronectin associates closely with the SEM proteins SEM1 and SEM2 to form the semen coagulum, a viscous gel that forms immediately post-ejaculation, we proceeded to test whether seminal fibronectin can enhance HIV infection. Prior literature suggested that *in vitro* polymerized fibronectin, called “superfibronectin,” efficiently enhances HIV infection (12). We confirmed that superfibronectin, and not fibronectin, efficiently enhanced HIV infection of target cells (Fig. 1A). Furthermore, partial immunodepletion of fibronectin (Fig. 1B) decreased the ability of seminal fluid to enhance HIV infection (Fig. 1C). In contrast, depletion of other abundant seminal proteins such as lactoferrin and albumin did not significantly decrease the ability of semen to enhance HIV infection (Fig. 1B and 1C). Together, these results suggest that semen fibronectin occurs in a polymerized state that, like superfibronectin, enhances HIV infection. As the

fibronectin III repeat domain can physically bind to amyloid fibrils (13), we are interested in determining whether SEM fibrils can bind fibronectin and whether the two components can synergistically enhance HIV infectivity in this manner.

Aim 1

This aim focused on characterizing the genetic and environmental factors underlying the variability in infectivity-enhancing activity in semen samples from different donors. As discussed in the first Quarterly Report, our early analysis of 5 semen samples from 5 healthy donors, as well as 14 semen samples from one donor, revealed that the viral enhancement activity varies markedly between semen samples obtained from the same donor. This observation is consistent with that recently reported by a different group (11). This longitudinal variability undermines the value of performing cross-sectional studies with individuals of different ethnic background and age. Because of these findings we requested and were granted a Change of Scope for Aim 1 of the proposal. In the revised Aim 1, we instead focus on semen samples from HIV-infected individuals within the Options Cohort at UCSF. Below we describe our progress in this area.

Semen samples from HIV-infected individuals enhance HIV infection, but relative enhancement activity inversely correlates with SEM levels

We tested twenty semen samples from individuals in the Options Cohort chronically infected with HIV-1. All semen samples enhanced HIV-1 infection of TZM-bl cells (data not shown), suggesting that the viral enhancing activity of semen is present in both HIV- and HIV+ individuals. We further assessed the relative levels of SEM fibrils in these samples by ELISA (the original plan of testing for SEVI levels was complicated by the fact that our SEVI antibody has lost reactivity over time, and we unfortunately have not been successful in developing new batches of this antibody). Interestingly, we observed an inverse correlation between enhancing activity and the levels of the SEM fibrils (data not shown). These data suggest that there may exist antiviral components in semen that work together with viral enhancing factors such as the semen amyloids; however the overall effect is still an enhancement of HIV infection in the presence of semen. Future directions will be to identify the anti-HIV factors in the semen samples from HIV-infected men. Such factors could include, for example, RANTES which can prevent the attachment of HIV-1 to its co-receptor CCR5.

Levels of SEM amyloids in semen samples from HIV-infected individuals directly correlate with endogenous viral load

In addition to measuring viral enhancing activity and the levels of SEM amyloids, we also measured endogenous viral load in semen samples from the Options cohort. Strikingly, we found that the levels of SEMs differ markedly between HIV-infected men, and that levels correlate directly with the semen viral load, independent of the blood HIV viral load (Fig. 2). We hypothesize that the semen HIV viral load is driven in part by the interaction of HIV with these semenogelin amyloid fibrils produced within the seminal vesicles. If correct, this model could

explain the marked variability in semen HIV viral load and infectivity that is observed among HIV-infected men.

Aim 2

In Aim 2, we proposed to characterize the ability of semen fibrils to activate innate immune responses. In this section, we describe progress in two areas: SEM fibril-mediated induction of pro-inflammatory cytokines, and the ability of semen fibrils to decrease epithelial tight junctions.

SEM amyloids induce inflammation

The ability of SEM amyloids to induce an inflammatory response was first examined. Whereas the ability of SEM amyloids to induce pro-inflammatory cytokine induction by monocyte-derived dendritic cells (MDDCs) was donor-dependent (data not shown), peripheral blood CD4+ T cells consistently elevated secretion of IL-6 in response to the amyloids (Fig. 3A). Seminal fluid could similarly induce the production of IL6 in CD4+ PBLs (Fig. 3B). Seminal fluid also induced IL6 in endometrial epithelial cells (Fig. 3C). Collectively, these data suggest that an inflammatory cytokine response is elicited in cells of the genital mucosa upon exposure to SEM amyloids or seminal fluid. Future directions include elucidating the signaling pathways activated in these cells.

Semen amyloids decrease epithelial tight junction integrity

Tight junctions between primary genital epithelial cells are disrupted by HIV exposure (14). Mechanistically, the HIV-1 envelope protein gp120 induces secretion of proinflammatory cytokines (e.g. TNFα) that impair barrier function. Semen may further reduce the integrity of tight junctions in the epithelial monolayer lining the genital mucosa, which could facilitate entry of HIV into the submucosa. To assess the ability of semen to decrease the integrity of a cervical epithelial monolayer, primary vaginal epithelial cells were cultured, and barrier integrity was measured by transepithelial resistance (TER). We found that seminal fluid decreased TER in a dose-dependent manner (Fig. 4A) under conditions that preserved cell viability (Fig. 4B). In contrast, boiled seminal fluid did not induce this response (Fig. 4A). Interestingly, SEVI fibrils had a modest ability to decrease TER in comparison to seminal fluid (Fig. 4A). The decrease in TER mediated by seminal fluid was coupled with a decrease in the expression of the tight junction proteins ZO-1 and occludin (Fig. 4C and 4D). These data suggest that semen may promote HIV transmission by decreasing epithelial barrier integrity. Future directions will be to determine whether semen synergizes with HIV to decrease barrier integrity, and to determine the molecular mechanisms underlying this phenomenon.

Aim 4

Aim 4 focuses on the identification of compounds that can inhibit the ability of semen to enhance HIV infection. A targeted approach and an unbiased approached were proposed.

Structural analogs of surfen were less potent than surfen in inhibiting the ability of semen to enhance HIV infection

We previously identified surfen as a small molecule that inhibits the ability of both SEVI and semen to enhance HIV infection (6). To determine whether modifying the structure of surfen will render it more effective, we tested surfen analogs for the ability to inhibit semen-mediated enhancement of HIV infection. We identified three structural analogs of surfen: 4-aminoquinaldine, 1,3-di-6-quinolylurea, and Scarlet acid. We found that although all of the analogs had some inhibitory activity against the ability of semen to enhance HIV infection, none of them were as potent as surfen (Fig. 5). As such, surfen remains the most potent compound we have identified that decreases semen's viral enhancing activity.

Small molecule screen for compounds that can inhibit the ability of semen to enhance HIV infection

We conducted a pilot screen of 2,688 bio-active compounds to identify small molecules that can inhibit the ability of semen to enhance HIV infection. We screened for compounds that could deaggregate the SEM fibril SEM1(49-107) (9) as assessed by Thioflavin T fluorescence. We identified 46 compounds that decreased the ThT emission of the SEM fibrils. These compounds were tested for the ability to quench fluorescence at ~482 nm, to eliminate false positive quenchers. Non-quenching compound hits are shown in Fig. 6. We are currently conducting secondary analysis to determine which of these 46 compounds decrease the ability of semen to enhance HIV infection. Preliminary data suggests that hematein may harbor such activity (data not shown). We have also initiated a full screen of 135,000 compounds for deaggregators, and will conduct secondary analysis of hits from this screen.

Aim 5

SEVI increases HIV transmission of MISTRG humanized mice

In collaboration with Drs. Shomyseh Sanjabi and Emily Deal, we obtained a colony of MISTRG humanized mice that were engrafted with human fetal liver. These mice were infected intravaginally 20 μl of 293T supernatant containing 1.6 ng of p24 of CCR5-tropic HIV-1 81A, in the absence or presence of 100 μg/ml of synthetic SEVI amyloid fibrils. Three days post-infection, spleens from the mice were harvested, stained for intracellular p24, and examined by flow cytometry. As shown in Fig. 7A, mice that were infected with HIV in the presence of SEVI had a higher percentage of p24+ CD4+ T cells. In addition, the addition of SEVI during infection increased the mean fluorescence of intracellular p24 in CD4+ T cells in HIV-infected mice (Fig. 7B). These preliminary results suggest that HIV-1 establishes infection in MISTRG mice, and that SEVI enhances this process. Future experiments to determine whether productive infection is occurring will incorporate controls in which HIV-1 is administered in the presence of the HIV-1 inhibitor AZT.

Key Research Accomplishments

- Identification of new factor from semen that enhances HIV infection. Identification and characterization of this factor was published in a manuscript entitled “Peptides released by physiological cleavage of semen coagulum proteins form amyloids that enhance HIV infection” (Cell Host and Microbe. 2011 10(6)541)

- Semen samples from HIV-infected men enhance HIV infection, and semen viral load in these samples correlates with the levels of semen amyloid

- Semen and semen fibrils induce an inflammatory response and decrease epithelial monolayer integrity

- Identification of semen amyloid deaggregators that decrease the ability of semen amyloids to enhance HIV infection

- The semen amyloid SEVI enhances vaginal transmission in a humanized mouse model of HIV infection

Reportable Outcomes

- Manuscripts
    - “Peptides released by physiological cleavage of semen coagulum proteins form amyloids that enhance HIV infection.”Roan NR, Müller JA, Liu H, Chu S, Arnold F, Stürzel CM, Walther P, Dong M, Witkowska HE, Kirchhoff F, Münch J, Greene WC. Cell Host Microbe. 2011 Dec 15;10(6):541-50. PMID: 22177559
    - [Role of semen-derived amyloid fibrils as facilitators of HIV infection]. Roan NR, Cavrois M, Greene WC. Med Sci (Paris). 2012 Apr;28(4):358-60. Epub 2012 Apr 25. French. PMID: 22549858

- Abstracts / Presentations
    - Roan N.R. Oral presentation at the 19th International AIDS Conference, Washington D.C., July 2012.
    - Roan N.R. “Identification and characterization of amyloid fibrils from human semen that enhance HIV infection”. Invited seminar at Symposium of the International Graduate School in Molecular Medicine, Ulm University. March 2012.
    - Roan N.R. “Identification and characterization of amyloid fibrils from human semen that enhance HIV infection”. Invited seminar at Jiaotong University School of Medicine, Shanghai, China. November 2011.
    - Roan N.R. “Identification and characterization of amyloid fibrils from human semen that enhance HIV infection”. Invited seminar at

University of California, Berkeley, Infectious Diseases and Immunity seminar series. October 2011.

- Funding acquired based on work
    - 1 R01 HD074511 - 01 (P0047347) (NIH-NICHHD)

Conclusions

The overall goal of this proposal is to better understand how semen and semen amyloids promote HIV transmission, and to identify ways to block this activity. In the first year of our funding, we have succeeded in identifying and characterizing novel viral enhancing factors from semen. We studied the ability of individual enhancing factors as well as semen in its entirety to enhance HIV infection directly, or indirectly by promoting inflammation and a breach in the primary epithelial cell monolayer. We further studied these factors in semen samples from HIV-infected men, and provided evidence that semen amyloids can promote HIV transmission *in vivo.* The practical application of understanding the activity of these amyloids is to identify novel ways to prevent HIV transmission. To this end, we have initiated a small molecule screen for inhibitors of the viral enhancing activity of semen, and have identified some promising candidates. Future directions will be to test the ability of the candidates to block transmission *in vivo*, and to study their effects on cells within the genital mucosa. Such knowledge will be crucial for the eventual development of candidate hit compounds into anti-HIV microbicides.

References

1. Abdool Karim SS, Richardson BA, Ramjee G, Hoffman IF, Chirenje ZM, Taha T, Kapina M, Maslankowski L, Coletti A, Profy A, Moench TR, Piwowar-Manning E, Masse B, Hillier SL, Soto-Torres L. (2011) Safety and effectiveness of BufferGel and 0.5% PRO2000 gel for the prevention of HIV infection in women. *Aids* **25**:957-966. 3083640
2. Bouhlal H, Chomont N, Haeffner-Cavaillon N, Kazatchkine MD, Belec L, Hocini H. (2002) Opsonization of HIV-1 by semen complement enhances infection of human epithelial cells. *J Immunol* **169**:3301-3306.
3. Munch J, Rucker E, Standker L, Adermann K, Goffinet C, Schindler M, Wildum S, Chinnadurai R, Rajan D, Specht A, Gimenez-Gallego G, Sanchez PC, Fowler DM, Koulov A, Kelly JW, Mothes W, Grivel JC, Margolis L, Keppler OT, Forssmann WG, Kirchhoff F. (2007) Semen-derived amyloid fibrils drastically enhance HIV infection. *Cell* **131**:1059-1071.
4. Hauber I, Hohenberg H, Holstermann B, Hunstein W, Hauber J. (2009) The main green tea polyphenol epigallocatechin-3-gallate counteracts semen-mediated enhancement of HIV infection. *Proc Natl Acad Sci U S A* **106**:9033-9038.
5. Roan NR, Munch J, Arhel N, Mothes W, Neidleman J, Kobayashi A, Smith-McCune K, Kirchhoff F, Greene WC. (2009) The cationic properties of

SEVI underlie its ability to enhance human immunodeficiency virus infection. *J Virol* **83**:73-80.
6. Roan NR, Sowinski S, Munch J, Kirchhoff F, Greene WC. (2010) Aminoquinoline surfen inhibits the action of SEVI (semen-derived enhancer of viral infection). *J Biol Chem* **285**:1861-1869.
7. Kim KA, Yolamanova M, Zirafi O, Roan NR, Staendker L, Forssmann WG, Burgener A, Dejucq-Rainsford N, Hahn BH, Shaw GM, Greene WC, Kirchhoff F, Munch J. (2010) Semen-mediated enhancement of HIV infection is donor-dependent and correlates with the levels of SEVI. *Retrovirology* **7**:55.
8. Olsen JS, Brown C, Capule CC, Rubinshtein M, Doran TM, Srivastava RK, Feng C, Nilsson BL, Yang J, Dewhurst S. (2010) Amyloid-binding small molecules efficiently block SEVI (semen-derived enhancer of virus infection)- and semen-mediated enhancement of HIV-1 infection. *J Biol Chem* **285**:35488-35496.
9. Roan NR, Muller JA, Liu H, Chu S, Arnold F, Sturzel CM, Walther P, Dong M, Witkowska HE, Kirchhoff F, Munch J, Greene WC. (2011) Peptides Released by Physiological Cleavage of Semen Coagulum Proteins Form Amyloids that Enhance HIV Infection. *Cell host & microbe* **10**:541-550.
10. Arnold F, Schnell J, Zirafi O, Sturzel C, Meier C, Weil T, Standker L, Forssmann WG, Roan NR, Greene WC, Kirchhoff F, Munch J. (2011) Naturally Occurring Fragments from Two Distinct Regions of the Prostatic Acid Phosphatase Form Amyloidogenic Enhancers of HIV Infection. *Journal of virology*
11. Hartjen P, Frerk S, Hauber I, Matzat V, Thomssen A, Holstermann B, Hohenberg H, Schulze W, Schulze Zur Wiesch J, van Lunzen J. (2012) Assessment of the range of the HIV-1 infectivity enhancing effect of individual human semen specimen and the range of inhibition by EGCG. *AIDS research and therapy* **9**:2.
12. Greco G, Pal S, Pasqualini R, Schnapp LM. (2002) Matrix fibronectin increases HIV stability and infectivity. *J Immunol* **168**:5722-5729.
13. Cisse M, Halabisky B, Harris J, Devidze N, Dubal DB, Sun B, Orr A, Lotz G, Kim DH, Hamto P, Ho K, Yu GQ, Mucke L. (2011) Reversing EphB2 depletion rescues cognitive functions in Alzheimer model. *Nature* **469**:47-52. 3030448
14. Nazli A, Chan O, Dobson-Belaire WN, Ouellet M, Tremblay MJ, Gray-Owen SD, Arsenault AL, Kaushic C. (2010) Exposure to HIV-1 directly impairs mucosal epithelial barrier integrity allowing microbial translocation. *PLoS Pathog* **6**:e1000852.

Appendix

Attached to the appendix is a copy of our manuscript “Peptides Released by Physiological Cleavage of Semen Coagulum Proteins Form Amyloids that Enhance HIV Infection”, published last year in Cell Host and Microbe (**10**:541-550). The Department of Defense is acknowledged therein as a funding source.

Cell Host & Microbe
Article

Peptides Released by Physiological Cleavage of Semen Coagulum Proteins Form Amyloids that Enhance HIV Infection

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SUMMARY

Semen serves as a vehicle for HIV and promotes sexual transmission of the virus, which accounts for the majority of new HIV cases. The major component of semen is the coagulum, a viscous structure composed predominantly of spermatozoa and semenogelin proteins. Due to the activity of the semen protease PSA, the coagulum is liquefied and semenogelins are cleaved into smaller fragments. Here, we report that a subset of these semenogelin fragments form amyloid fibrils that greatly enhance HIV infection. Like SEVI, another amyloid fibril previously identified in semen, the semenogelin fibrils exhibit a cationic surface and enhance HIV virion attachment and entry. Whereas semen samples from healthy individuals greatly enhance HIV infection, semenogelin-deficient semen samples from patients with ejaculatory duct obstruction are completely deficient in enhancing activity. Semen thus harbors distinct amyloidogenic peptides derived from different precursor proteins that commonly enhance HIV infection and likely contribute to HIV transmission.

INTRODUCTION

Sexual transmission accounts for the vast majority of new HIV infections, and semen is the vehicle fueling the global spread of this retrovirus. We and others have demonstrated that semen enhances in vitro HIV infection of multiple cell types, including primary macrophages and CD4+ T cells, two biologically relevant targets of HIV in vivo (Kim et al., 2010; Münch et al., 2007; Olsen et al., 2010; Roan et al., 2009). Fractionation of a peptide library generated from semen led to the identification of peptides derived from prostatic acid phosphatase (PAP) that self-assembled into amyloid fibrils that greatly enhanced HIV infection under conditions of limiting viral innocula (Münch et al., 2007). The amyloids were termed semen-derived enhancers of viral infection (SEVI).

We previously showed that SEVI enhances HIV infection by promoting viral attachment to target cells (Münch et al., 2007; Roan et al., 2009). SEVI is highly cationic (8 of its 39 residues are positively charged at neutral pH), and the positive charges of SEVI interact directly with the negatively charged surfaces of target cells and HIV virions to promote attachment and fusion. When the cationic property of SEVI is abrogated by site-directed mutagenesis or by adding anionic polymers, SEVI loses its ability to augment HIV infection (Roan et al., 2009). Importantly, anionic polymers that inhibit the infection-enhancing activity of chemically synthesized SEVI also inhibit the enhancing activity of semen (Roan et al., 2009), suggesting that endogenous SEVI or other positively charged factors in semen play a role in its ability to enhance viral infection.

We sought to confirm the role of positively charged factors for the ability of semen to enhance HIV infection by depleting these factors. In line with our previous data using anionic polymers (Roan et al., 2009), semen depleted of positively charged factors lost the ability to enhance HIV infection. Surprisingly, when we identified the cationic factors that were specifically depleted, we detected not SEVI but, rather, fragments from the semenogelins (SEMs), which are abundant proteins in semen that together with fibronectin constitute the semen coagulum, a gelatinous structure crucial for fertilization (de Lamirande, 2007; Robert and Gagnon, 1999). We further pursued this finding and describe here the identification of peptides from SEMs that form amyloids that enhance HIV infection. We further demonstrate that variations in SEM levels directly correlate with enhancement of HIV infection and that semen samples naturally lacking SEMs are completely deficient in this effect. Altogether, our data suggest that SEMs may play a previously unrecognized role in promoting HIV infection in semen, thus indicating that factors whose physiological purpose is to promote fertilization may unfortunately also promote HIV transmission.

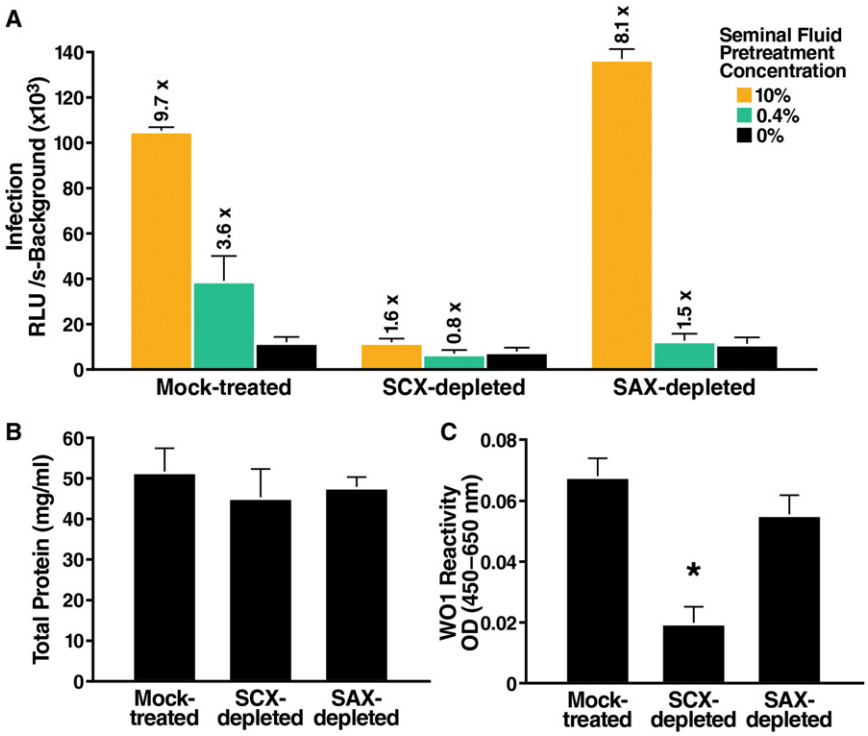


Figure 1. Seminal Fluid Depleted of Cationic Factors Lacks Viral Enhancement Activity

(A) Seminal fluid that was mock-treated, depleted of cationic factors (SCX-depleted), or depleted of anionic factors (SAX-depleted) was tested for the ability to enhance HIV infection. 81A virions were pretreated for 5 min with the indicated concentrations of seminal fluid and then diluted 15-fold and added to TZM-bl cells. Medium was replaced after 2 hr and cells were assayed for Tat-inducible β-galactosidase activity 3 days later. The numbers above the bars indicate the n-fold infectivity enhancement relative to infection measured in the absence of seminal fluid. RLU/s: relative light units/second. Shown are average values (± standard deviations) of triplicate measurements from one of three independent experiments that yielded similar results. See also Figure S1.

(B) The total protein content of mock-treated, SCX-depleted, or SAX-depleted seminal fluid was assessed by the BCA assay. Shown are average values (± standard deviations) of triplicate well measurements from one of two independent experiments that yielded similar results.

(C) Mock-treated, SCX-depleted, or SAX-depleted seminal fluid was tested for relative reactivity to WO1, an amyloid-specific antibody, by ELISA. The reactivity of WO1 to PBS was subtracted from all values. The OD (450–650 nm) indicates the relative levels of WO1-reactive amyloids in each sample. *$p < 0.005$ versus mock-treated or SAX-depleted seminal fluid (two-tailed t test). Shown are average values (± standard deviations) of triplicate well measurements from one of two independent experiments that yielded similar results.

RESULTS

Seminal Fluid Depleted of Cationic Factors Is Deficient in Enhancing HIV Infection

We previously demonstrated that the anionic polymers heparin and dextran sulfate, but not less negatively charged polymers such as chondroitin sulfate, inhibit the ability of semen to enhance HIV infection. Oversulfation of chondroitin sulfate converted this polymer into a potent inhibitor, suggesting that anionic polymers with higher concentrations of negative charges are more effective in inhibiting semen's ability to enhance HIV infection (Roan et al., 2009). These results suggest that positively charged factors are responsible for the viral infection enhancing activity of semen.

To further test this hypothesis, we depleted cationic (i.e., positively charged) factors from seminal fluid (pooled from 20 donors) using strong cation exchange (SCX) beads. As negative controls, we mock-depleted seminal fluid or depleted anionic (i.e., negatively charged) factors with strong anion exchange (SAX) beads. Depletion of positively charged factors, but not negatively charged factors, abrogated the ability of semen to enhance HIV infection of TZM-bl reporter cells (Figure 1A). Total protein content was similar in the SCX- and SAX-depleted seminal fluid (Figure 1B), suggesting that the absence of viral-enhancing activity in SCX-depleted seminal fluid was not due to the absence of protein.

Lack of Semenogelin Fragments in Seminal Fluid Depleted of Cationic Factors

To identify the factors that were enriched in the SAX-depleted seminal fluid (which enhanced HIV infection) relative to the SCX-depleted seminal fluid (which did not enhance HIV infection), we employed mass spectrometry. In two independent experiments, matrix-assisted laser desorption ionization /tandem mass spectrometry (MALDI MS/MS) detected exclusively fragments from SEM1 and SEM2 (Table S1A), highly homologous proteins in semen that interact with fibronectin to form the semen coagulum. This coagulum is progressively cleaved by the protease prostate specific antigen (PSA) at specific sites within the SEMs during semen liquefaction (Robert et al., 1997). The SEM peptides identified by MALDI MS-MS included PSA-generated proteolytic products (Robert et al., 1997) (Table S1A). Although PAP248–286 or related fragments known to form SEVI were not detected by MALDI MS/MS, when assessed by ELISA, PAP248–286 was partially depleted by the SCX beads (Figure S1). The depletion of SEM1 and SEM2 fragments by SCX is not surprising, as both of these basic proteins are positively charged at neutral pH. Because the SEMs were deficient in seminal fluid depleted of viral-enhancing activity, we pursued the possibility that SEMs, like SEVI, might correspond to natural enhancers of HIV infection.

Semenogelin Fragments Form Amyloid Fibrils

We first investigated whether the SEMs were capable of forming amyloid fibrils, with the rationale that if SEMs enhance HIV infection like SEVI, they may also do so as an amyloid fibril. Although to our knowledge no peptides from SEM have been directly demonstrated to form amyloid fibrils, antibodies raised against an N-terminal region of SEM1 (SEM1[45–64]) recognize fibrillar structures isolated from the seminal vesicles of patients with senile seminal vesicle amyloid (SSVA), one of the most

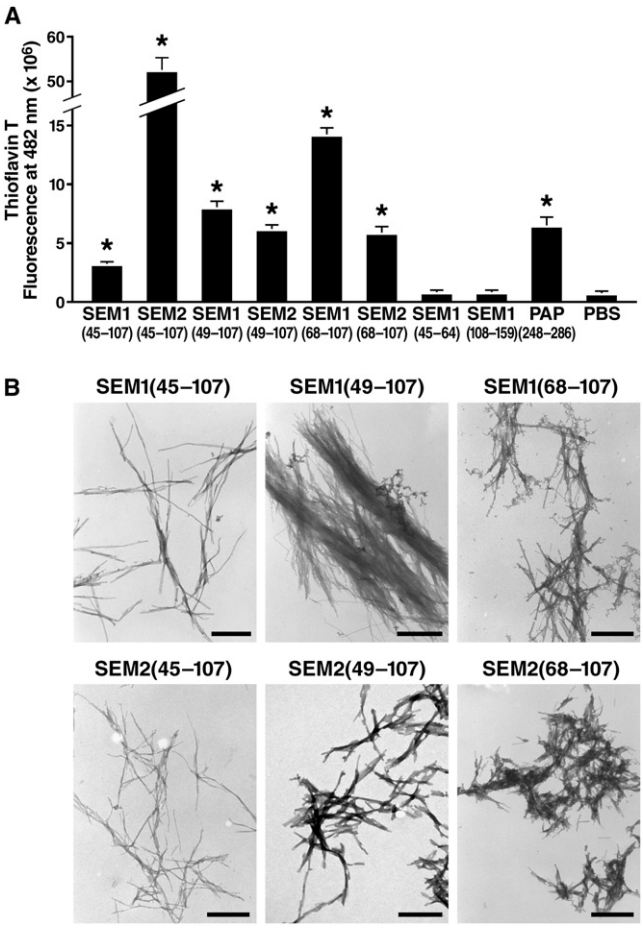


Figure 2. Peptides from SEM1 and SEM2 Form Amyloid Fibrils

(A) The indicated that agitated peptides were mixed with 5 μM thioflavin T, and the emissions at 482 nm were recorded. PAP248–286 is an amyloidogenic peptide that polymerizes into SEVI (Münch et al., 2007). *$p < 0.0005$ versus PBS control (two-tailed t test). Shown are average values (± standard deviations) of triplicate well measurements from one of seven independent experiments that yielded similar results. See Table S1 for peptide sequences. (B) Electron micrographs of the fibrillar SEM peptides. Scale bars indicate 500 nm. Shown are results from one of two independent experiments that yielded similar results.

common forms of localized amyloidosis (Linke et al., 2005). Because full-length SEMs are rare in semen due to the efficiency of PSA cleavage (Koistinen et al., 2002; Robert et al., 1997), an amyloid form of the SEMs in semen would likely be composed of SEM fragments. To determine if we could identify amyloidogenic fragments from semen, we took advantage of WO1, an amyloid-specific antibody that recognizes the general amyloid fold in a sequence-independent manner (O'Nuallain and Wetzel, 2002). WO1 had less reactivity to SCX-depleted seminal fluid compared to mock-treated or SAX-depleted seminal fluid (Figure 1C), suggesting a relative deficiency of amyloids in seminal fluid depleted of cationic factors. Another amyloid-specific antibody, OC (Kayed et al., 2007), also showed higher reactivity to SAX-depleted seminal fluid compared to SCX-depleted seminal fluid (data not shown). To identify the amyloid species, we conjugated WO1 to magnetic beads and incubated them with seminal fluid. Bead-bound material was digested with trypsin and then identified by liquid chromatography (LC) MALDI MS/MS. In three independent experiments, we identified fragments from the N-terminus of the SEM proteins in WO1 samples and not from control samples, suggesting that SEM amyloids might be present in semen. Interestingly, the identified peptides were immediately C-terminal to SEM1(45–64), the region that was suggested as amyloidogenic in SSVA patients (Linke et al., 2005). We chemically synthesized a peptide corresponding to SEM1(45–64) but found no evidence that it formed amyloid fibrils when tested under multiple conditions (data not shown). Because we had detected tryptic peptides immediately downstream of SEM1(45–64), we reasoned that the region C-terminal of SEM1(45–64) might be important for amyloid fibril formation. Therefore, we chemically synthesized three peptides harboring sequences C-terminal of SEM1(45–64): SEM1(45–107), SEM1(49–107), and SEM1(68–107). The junctions following residues 49 and 68 were chosen because they correspond to natural PSA cleavage sites within SEM1 (Robert et al., 1997). As such, peptides cleaved at these sites are likely to be present in liquefied semen. The corresponding highly homologous SEM2 peptides were also synthesized (Table S1B).

After overnight agitation at 37°C to promote fibril formation, SEM1(45–107), SEM2(45–107), SEM1(49–107), SEM2(49–107), SEM1(68–107), and SEM2(68–107) all formed amyloid fibrils, whereas SEM1(45–64) and a further downstream peptide, SEM1(108–159), did not. Evidence for fibril formation included an increase in the fluorescence intensity of thioflavin T (Figure 2A), binding of Congo red dye (data not shown), and detection of typical thread-like fibrillar structures by electron microscopy analyses (Figure 2B). Electron micrographs of the SEM fibrils, together with HIV virions, demonstrated extensive interactions between the two components (Figure 3A); these results were further verified by fluorescence microscopy (Figure 3B). Three-color imaging of the fibrils, virions, and cells suggested that the fibrils promoted attachment of the virions to target cells (Figure S2). Thus, peptides harboring sequences immediately C-terminal of SEM1(45–64), as well as SEM2 peptide homologs, are capable of forming amyloid fibrils and interacting with virions and cells.

Semenogelin Fibrils Enhance HIV Infection

We next tested whether these amyloid fibrils enhance HIV infection. Like SEVI, the SEM fibrils are rich in basic residues and, thus, exhibit a net positive charge at neutral pH; isoelectric points of the SEM peptides used in this study range from 8.16 to 10.12, as indicated in Table S1B. Because SEVI enhances HIV fusion by promoting attachment of HIV virions to target cells (Münch et al., 2007; Roan et al., 2009), we tested whether SEM fibrils act in a similar manner. The six SEM peptides that form fibrils enhanced fusion of CCR5-tropic HIV-1 81A virions to primary CD4+ T cells (Figure 4A). In contrast, SEM1(45–64) and SEM1(108–159), which fail to form fibrils (Figure 2A), did not enhance viral fusion. Similar to SEVI, the enhancing effect of the SEM fibrils was not coreceptor-specific, as fusion of CXCR4-tropic HIV-1 NL4-3 to CD4+ T cells was similarly enhanced (Figure 4B). Interestingly, the fibrils that were composed of shorter peptides, SEM1(68–107) and SEM2(68–107), enhanced fusion to a lesser extent than the fibrils formed with

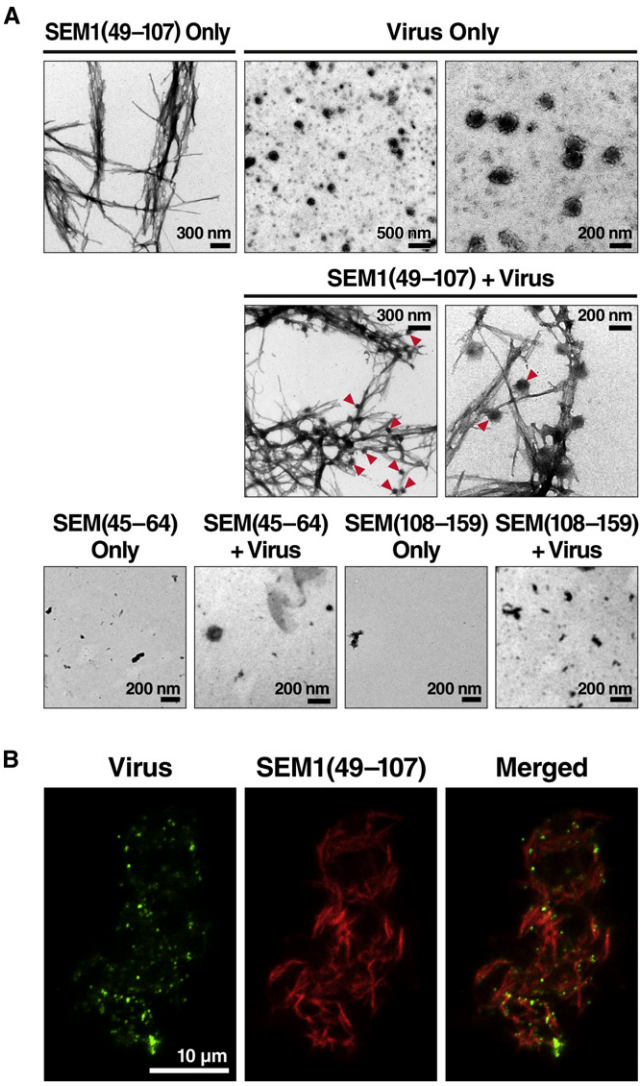


Figure 3. Visualization of Viral Particles Bound to SEM Fibrils

(A) Electron micrographs of SEM1(49–107) fibrils, HIV viral particles, and SEM1(49–107) fibrils incubated together with the viral particles. Arrowheads indicate examples of the sites of contact of virions with fibrils. Nonfibrillar SEM1(45–64) and SEM1(108–159) were used as negative controls. Shown are results from one of two independent experiments that yielded similar results. (B) Fluorescence microscopy of SEM1(49–107) fibrils (red) and MLV-gag-YFP viral particles (green). Shown are results from one of two independent experiments that yielded similar results. See also Figure S2.

the longer peptides, SEM1(45–107), SEM2(45–107), SEM1(49–107), and SEM2(49–107).

Similar to SEVI (Roan et al., 2009), the enhancing effect of the SEM fibrils was dependent on their cationic property; using the anionic polymer heparin to shield the fibrils' positive charges abrogated their viral-enhancing activities (Figure S3). Furthermore, when surface-exposed positive charges of the fibrils were measured by zeta potential, we found that the SEM1(45–107), SEM2(45–107), SEM1(49–107), and SEM2(49–107) fibrils exhibited high potentials, whereas the two fibrils made up of the shorter peptides, SEM1(68–107) and SEM2(68–107), exhibited low potentials (Table S1B). This decreased zeta potential may explain why the SEM1(68–107) and SEM2(68–107) fibrils enhanced HIV fusion less potently than the other four. In support of this possibility, the zeta potentials of the fibrils correlated directly with the relative ability to enhance HIV fusion to target cells (Figure S5A).

To determine if the SEM fibrils enhance productive infection, we infected TZM-bl cells with an R5-tropic HIV-1 (Papkalla et al., 2002) in the presence of increasing concentrations of the fibrils. Each of the six SEM fibrils enhanced HIV infection (Figure 5). Although the relative levels of viral enhancement between the different SEM fibrils varied between experiments, in general, we observed that the fibrils derived from the shorter peptides, SEM1(68–107) and SEM2(68–107), were less effective at enhancing HIV infection. Finally, we demonstrated that SEM-derived fibrils were capable of rescuing the viral-enhancing activity of SCX-depleted seminal fluid (Figure S4).

Levels of Semenogelin Correlate with HIV-Enhancing Activity in Semen Samples from Different Donors

To develop tools to assess endogenous levels of SEM fragments, we generated polyclonal antibodies against fibrils formed by the two fragments, SEM1(49–107) and SEM2(49–107). Anti-SEM1(49–107) (hereafter called anti-S1) recognized SEM1(49–107) in a dose-dependent manner and anti-SEM2 (49–107) (hereafter called anti-S2) recognized SEM2(49–107) in a dose-dependent manner (Figure S5B). The antibodies were not fibril-specific because they recognized the monomeric and fibrillar forms of the peptides to similar extents (data not shown). When we compared the reactivities of anti-S1 and anti-S2 to a standard curve, we found that the levels of reactivity against the antisera in semen samples from 20 individuals corresponded to concentrations of antigen of 90–3,000 μg/ml, well above the range necessary for enhancing HIV infection.

We tested the relative ability of semen samples from 20 individuals to enhance HIV-1 infection of TZM-bl cells and plotted this as a function of the relative levels of SEM1- and SEM2-containing fragments as assessed using anti-S1 and anti-S2. The relative abilities to enhance HIV infection correlated directly with levels of SEM1 and SEM2 (Figure 6A). Consistent with published results (Kim et al., 2010), we also observed a direct correlation between the levels of PAP248–286/SEVI and enhancing activity (Figures 6B and S6C). In contrast, the levels of the seminal proteins albumin, lactoferrin, PSA, and full-length PAP did not correlate with viral-enhancing activity. Finally, a statistically significant direct correlation existed between viral-enhancing activity and reactivity to the antibodies WO1 and WO2, which recognize the general amyloid fold (O'Nuallain and Wetzel, 2002) (Figures 6B and S5C). Taken together, these results support roles for both SEM and SEVI amyloids in semen-mediated enhancement of viral infection.

Semen Samples Naturally Deficient in Semenogelin Do Not Enhance HIV Infection

Semen samples from patients with ejaculatory duct obstruction (EDO) have low levels of SEMs because secretions from the seminal vesicles do not effectively reach the ejaculate (Edström et al., 2008; Pryor and Hendry, 1991). If SEMs are important

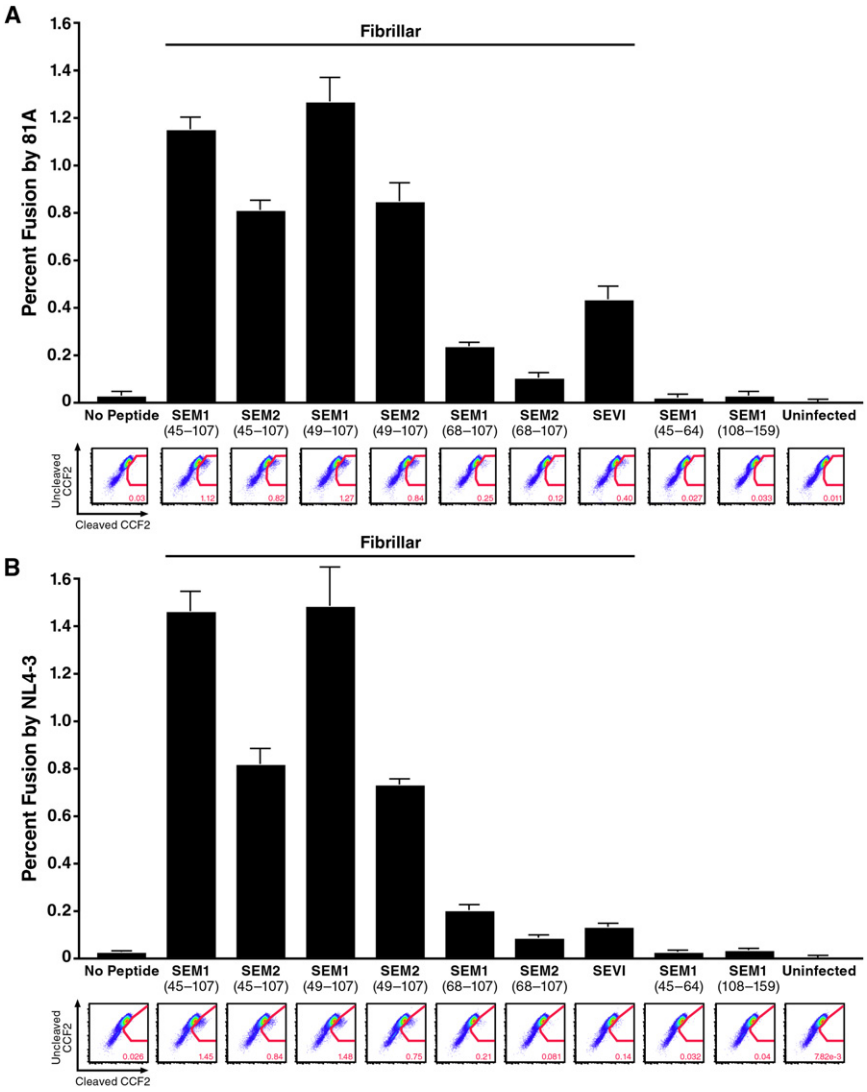


Figure 4. SEM Fibrils Enhance Fusion of Both CCR5- and CXCR4-Tropic HIV-1 to Primary CD4+ T Cells

CD14-CD4+ T cells were infected with BlaM-Vpr-containing 81A (A) or NL4-3 (B) virions pretreated with 31.25 μg/ml of the indicated agitated peptides. Peptides that formed fibrils (Figure 2) are indicated. Shown are the averages of triplicate samples with a representative FACS plot presented directly below. Similar results were obtained using cells from three other donors. See also Figure S3.

for the viral infection-enhancing activity of semen, then semen from EDO patients should be deficient in this activity. Five semen samples from EDO patients were tested for the ability to enhance HIV-1 81A infection of TZM-bl cells. All five samples from EDO patients completely lacked HIV enhancement activity, in contrast to semen pooled from 20 normal donors (Figure 7A).

We used western blots to confirm the lack of SEM in the EDO samples. Commercially available antibodies against SEM1/2, as well as our anti-S1 and anti-S2 antibodies, detected SEM fragments in semen from healthy donors (Figure 7B). The predominance of multiple SEM fragments in semen is consistent with prior reports that PSA cleaves SEMs at multiple sites immediately post-ejaculation (Koistinen et al., 2002; Robert et al., 1997). In contrast to semen from healthy donors, samples from EDO patients had very little reactivity to the SEM antibodies (Figure 7B). Albumin levels were uniform across the samples, suggesting the EDO samples were not simply deficient in all proteins. Interestingly, the levels of PSA and PAP were higher in EDO samples, possibly from over-representation of prostate-derived proteins in semen from these patients (Figure 7B). These western blot results were confirmed by ELISA (data not shown). When we assessed the levels of the SEVI precursor peptide PAP248–286, we found that, whereas the levels of full-length PAP were increased in EDO samples (Figure 7B), the levels of PAP248–286 were decreased (Figure S6). These data suggest that the proteolytic activity necessary to generate SEVI from PAP may be decreased in semen from EDO patients. In conclusion, semen samples naturally deficient in secretions from the seminal vesicles lack the ability to enhance HIV infection.

DISCUSSION

Here, we identify a set of amyloidogenic peptides derived from SEMs, the major components of the semen coagulum. These SEM-derived amyloid fibrils join SEVI as semen factors that enhance virion attachment and entry into target cells. We further demonstrate that endogenous SEMs contribute greatly toward the viral-enhancing activity of semen by showing that (1) the relative enhancing activity of semen samples from different donors correlates with SEM levels, and (2) SEM-deficient semen from EDO patients lack viral-enhancing activity.

EDO patients, whose semen lacks secretions from the seminal vesicles, represent a small subset of infertility cases. We found that semen from these patients is deficient not only in SEMs but also in SEVI. Because semen from EDO patients harbor higher levels of full-length PAP (the precursor for SEVI), we suspect that the protease responsible for cleaving full-length PAP into the SEVI peptide may reside in the seminal vesicles. The identity of this protease remains undetermined. Because EDO semen samples are deficient in both SEM and SEVI amyloids, it is unclear which species represents the dominant enhancing activity of semen. Although it is unknown whether SEVI or SEM amyloids contribute more toward the viral-enhancing activity of semen, it is noteworthy that SEMs are more abundant than PAP. While the concentration of full-length SEM proteins is estimated at 10–20 mg/ml in semen (Yoshida

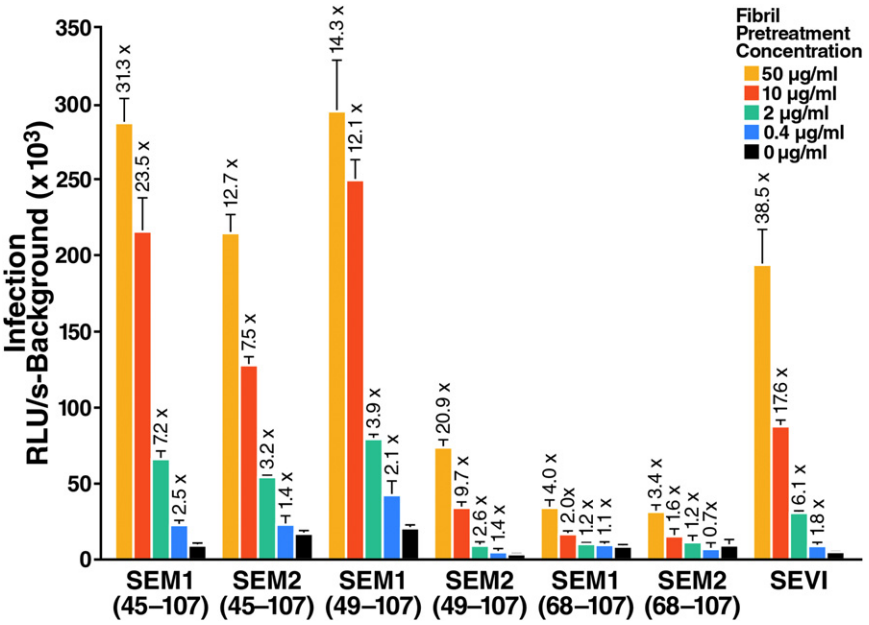


Figure 5. SEM Fibrils Enhance Productive Infection of Target Cells by HIV-1

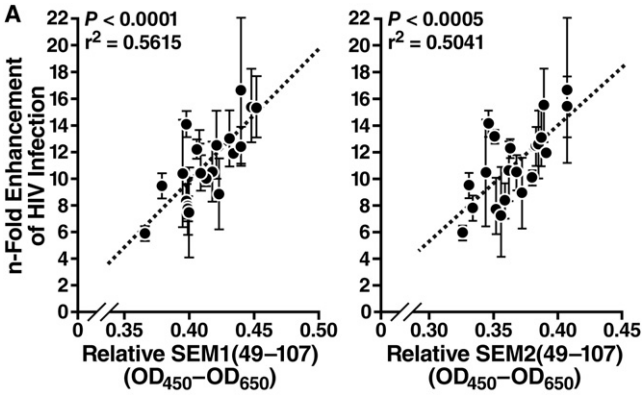
Virions were pretreated for 5 min with the indicated concentrations of SEM fibrils and used to infect TZM-bl cells. Cells were assayed for Tat-inducible β-galactosidase activity 2 days later. The numbers above the bars indicate the n-fold infectivity enhancement relative to infection measured in the absence of peptide. RLU/s: relative light units/second. Shown are average values (± standard deviations) of triplicate measurements from one of three independent experiments that yielded similar results. See also Figure S4.

et al., 2003), the concentration of PAP is only 1–2 mg/ml (Rönnberg et al., 1981). Failure of the amyloid-specific WO1 antibody to pull out SEVI from seminal fluid may be due to a higher abundance of the SEM proteins. We have confirmed that WO1 recognizes synthetic SEVI amyloid fibrils but not the monomeric PAP(248–286) peptide (data not shown). However, it is also possible that WO1 may not detect endogenous SEVI in semen, particularly if natural SEVI exists mainly as small protofibrils because amyloid-specific antibodies often recognize epitopes found in mature fibrils but not prefibrillar oligomers (Kayed et al., 2007). Furthermore, because amyloids can adopt a wide diversity of structures (reviewed by Toyama and Weissman [2011]), WO1 may not recognize all the different forms of SEVI that may exist in semen. To directly assess the relative contribution of SEVI and SEM fibrils for the enhancing activity of semen, we are developing conformation-specific monoclonal antibodies that can selectively immunodeplete these fibrils from semen. Regardless of which factor contributes more, a key prediction is that HIV-infected patients with EDO will be less likely to sexually transmit HIV because semen from EDO patients completely lacks the ability to enhance HIV infection. Testing this hypothesis would be interesting, albeit technically challenging, because EDO patients are estimated to represent only 1–5% of all male infertility cases (Pryor and Hendry, 1991).

We and others observed that semen enhances HIV infection in vitro (Bouhlal et al., 2002; Hauber et al., 2009; Kim et al., 2010; Münch et al., 2007; Olsen et al., 2010; Roan et al., 2009, 2010), supporting a potential role for SEM fibrils and SEVI in enhancing HIV infection during sexual transmission. In contrast, others have reported that semen inhibits HIV infection (Balandya et al., 2010; Martellini et al., 2009; Sabatté et al., 2007). These contrasting results may reflect multiple factors, including the presence of both enhancing and inhibitory factors for HIV infection, inhibitory effects due to semen-mediated toxicity on target cells, and different experimental conditions among laboratories. Notably, the inhibitory activity reported by Sabatté et al. was specific for DC-SIGN-dependent enhancement of trans-infection to T cells (Sabatté et al., 2007); indeed, we previously confirmed the presence of this inhibitory activity (Kim et al., 2010). Conversely, our observation that semen enhances direct HIV infection of multiple cell types stands in sharp contrast with reports that semen inhibits HIV infection in vitro (Balandya et al., 2010; Martellini et al., 2009). We have carefully reproduced the conditions used by Martellini and coworkers, who suggested that semen may inhibit HIV infection. Our analysis reveals a direct correlation between semen-induced cytotoxicity and the observed viral inhibitory activity of semen, suggesting that the observed inhibition was likely due to low viability of target cells (Kim et al., 2010). More recently, Balandya et al. reported that seminal fluid inhibits HIV infection by down-modulating a whole panel of cell-surface receptors (Balandya et al., 2010). Because they were working with a concentration of seminal fluid that is just at the threshold of killing target cells, we believe that cytopathic effects of the seminal fluid likely accounted for the observed inhibition of HIV infection. Indeed, their observation of a general decrease in all cell-surface receptors except CCR5, whose expression is increased on late apoptotic cells (Ariel et al., 2006), suggests that the target cells they used may have been undergoing apoptosis at the time of infection. Furthermore, we have found that inhibitory semen concentrations utilized by Balandya et al. are markedly cytotoxic for PBMCs (unpublished data). Recognizing that the in vitro cytotoxic effects of semen can complicate its evaluation, we have worked out and described methods for examining the effect of semen on HIV infection under conditions that preserve the viability of target cells (Kim et al., 2010; Münch et al., 2007). We believe it is paramount to carry out semen analysis under such nontoxic conditions because viral gene expression is dependent on the fitness of cells and cytotoxic effects may result in misleading conclusions.

Ultimately, the inhibitory or enhancing effect of semen on HIV infection requires careful assessment in in vivo models of mucosal HIV infection. Two studies have examined the effect of semen on vaginal infection of rhesus macaques with SIV. Miller et al. observed that human seminal plasma increased the rate of transmission at low but not high levels of SIV innocula (Miller et al., 1994). A second study reported increased vaginal

A



B

Viral Enhancing Activity

Directly Correlated with Reactivity to:	Not Directly Correlated with Reactivity to:
SEM1(49–107) Sera ($P < 0.0001$; $r^2 = 0.5615$)	**Pre-Imm** Sera
SEM2(49–107) Sera ($P < 0.0005$; $r^2 = 0.5041$)	**Albumin** Sera
PAP(248–286) Sera ($P = 0.0027$; $r^2 = 0.3837$)	**Lactoferrin** Sera
WO1 (Amyloid-specific) ($P = 0.0013$; $r^2 = 0.426$)	**PSA** Sera
WO2 (Amyloid-specific) ($P = 0.0319$; $r^2 = 0.2201$)	**PAP** (Full Length) Sera

Figure 6. The Magnitude of Enhancement of HIV-1 Infection by Individual Semen Samples Correlates Directly with the Relative Levels of SEM Peptides and PAP248–286

(A) Direct correlation between viral enhancement activity and the quantity of SEM1 (left) and SEM2 (right) fragments measured using the anti-S1 and anti-S2 sera, respectively. The n-fold enhancement on the y-axis was determined using a TZM-bl infectivity assay. Error bars reflect standard deviations of triplicate well measurements. The relative SEM levels on the x-axis were determined by ELISA. See also Figure S5B.

(B) Summary of all correlation analysis of viral-enhancing activity versus reactivity to the indicated antibodies. Semen samples from 20 donors were tested for relative viral-enhancing activity and for reactivity to the indicated antibodies. The left column displays cases where there was a direct correlation (p-values, all < 0.05, as shown) between viral-enhancing activity and reactivity to the indicated antibody. The right column displays cases where there was no direct correlation. See also Figure S5C.

SIVmac251 infection of macaques in the presence of semen (Neildez et al., 1998), again only under conditions of low viral inocula. Interestingly, the enhancing effects of SEVI and semen on HIV infection in vitro are also most prominent at low viral inocula (Münch et al., 2007). Unfortunately, in both of the in vivo studies mentioned, the small number of animals examined precluded meaningful statistical analysis. As such, the effect of semen in a low-dose vaginal challenge study powered with a sufficiently large number of animals to yield statistically significant results is urgently needed.

Previous studies support the notion that SEM peptides may form amyloids in vivo. Amyloid deposits reactive to SEM-specific antibodies have been observed in the seminal vesicles of patients with localized amyloidosis (Linke et al., 2005). Furthermore, localized amyloidosis of the seminal vesicles has been detected at a frequency of 17% in men over age 50 at autopsy (Bursell, 1942) and is thought to be a common condition (Pitkänen et al., 1983). The pathogenesis of such amyloids, if any, is unknown but our identification of SEM peptides that spontaneously form fibrils in vitro may provide insights into this condition.

Although the natural function of amyloid fibrils in semen, if any, is unknown, SEMs and their fragments have numerous roles in fertilization (de Lamirande, 2007; Robert and Gagnon, 1999). SEMs block sperm motility (Robert and Gagnon, 1996; Iwamoto and Gagnon, 1988) and prevent sperm capacitation (de Lamirande et al., 2001), thereby inhibiting premature release of active sperm. SEMs also activate sperm hyaluronidase, which helps degrade the egg envelope during fertilization (Mandal and Bhattacharyya, 1995). Cleavage of the SEMs by PSA during liquefaction is important for fertilization because men with semen with a slow liquefaction rate are infertile (Robert and Gagnon, 1995). In addition, several lines of evidence suggest that SEM fragments are associated with spermatozoa. SEM fragments have been reported to bind to the posterior head, midpiece, and tail of spermatozoa (Bjartell et al., 1996; de Lamirande, 2007), and antibodies raised against whole spermatozoa were found to recognize SEM1 (Herr et al., 1986). Interestingly, a SEM1 fragment was found within sperm nuclei, suggesting that SEM fragments may gain entry into these cells (Zalensky et al., 1993). Whether any of these reported activities of SEM or SEM fragments involve SEM-derived amyloid fibrils remains to be explored. Notably, spermatozoa are stainable with the amyloid-binding dye Congo red (Liu and Foote, 1998), suggesting the presence of one or more bound amyloid fibrils. It is certainly possible that SEM amyloids coat the surface of spermatozoa. This may, in turn, promote HIV binding to the spermatozoa and facilitate the transfer of virions to susceptible target cells in both the lower and upper reproductive tracts (Ceballos et al., 2009).

Notably, semen is thus far the only biological fluid that has been reported to contain multiple amyloidogenic peptides derived from abundant proteins. PAP and SEMs, the precursors for these amyloidogenic peptides, are proteins, whose expression is mostly limited to male reproductive organs. The existence of naturally occurring amyloidogenic peptides in semen may have an evolutionary role in promoting fertilization. Since SEM fibrils can increase HIV fusion, might they also have a role in fertilization by enhancing sperm-egg fusion? Many parallels exist between the fusion of HIV to cells and the fusion of sperm to egg (reviewed in Doncel [2006]). Both processes require lipid rafts and glycoproteins, and the molecular basis of the fusion processes share signaling pathways and involve integrins and tetraspanins. Furthermore, spermicidal compounds inhibit HIV infection, whereas virucidal compounds, such as polyanions and antimicrobial peptides, block sperm-egg fusion (Doncel, 2006). As we obtain a broader understanding of natural factors in semen that enhance HIV infection, we may gain additional insights into the molecular events that promote fertilization and

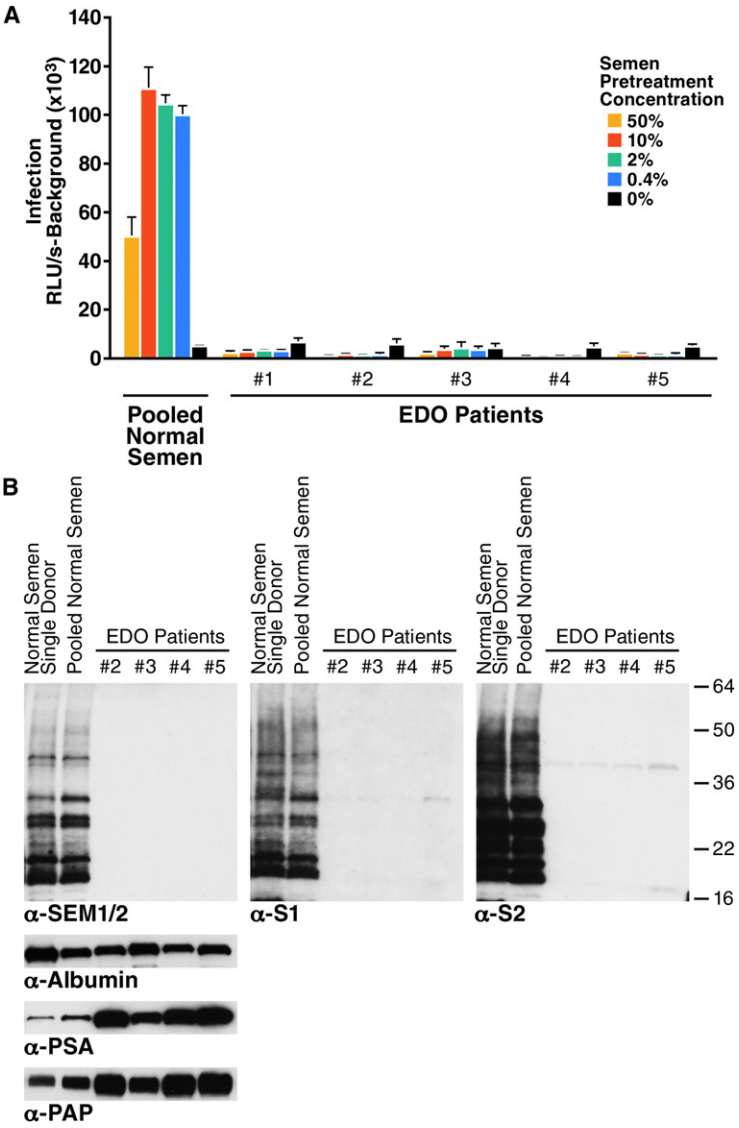


Figure 7. Semen Samples from EDO Patients Lack Viral-Enhancing Activity and Are Deficient in SEMs

(A) Pooled semen from normal donors or individual semen samples from patients with EDO were pretreated at the indicated concentrations with 81A virions for 5 min. Samples were then diluted 15-fold and added to TZM-bl cells. Medium was replaced after 2 hr, and cells were assayed for Tat-inducible β-galactosidase activity 3 days later. RLU/s: relative light units/second. Shown are average values (± standard deviations) of triplicate measurements. The decrease in infectivity enhancement with a semen pretreatment concentration of 50% is due to semen-mediated toxicity to the cells (Kim et al., 2010).

(B) Semen samples from normal donors or EDO patients were analyzed by western blot using commercial antibodies recognizing both SEM1 and SEM2 (α-SEM1/2), or the anti-S1 and anti-S2 antibodies generated for this study. Samples were also blotted for albumin, PSA, and PAP as indicated. Note that EDO1 was not analyzed by western blot analysis due to limited material. See also Figure S6.

the potential involvement of these same factors. Perhaps agents that block HIV infection by targeting viral-enhancing factors, such as semen-derived amyloid fibrils, might also serve as contraceptives.

EXPERIMENTAL PROCEDURES

Reagents

Custom peptides (synthesized by Celtek Peptides, Nashville, TN, and CPC Scientific, Sunnyvale, CA) were dissolved in PBS at concentrations of 2.5–10 mg/ml, and amyloid fibril formation was promoted by overnight agitation at 37°C at 1,400 rpm with an Eppendorf Thermomixer. Deidentified semen samples from healthy donors (obtained from the UCSF Fertility Clinic, San Francisco, CA) were allowed to liquefy for 2 hr at room temperature and then were frozen at −20°C. Seminal fluid was generated by centrifuging semen pooled from 20 donors for 30 min at 4°C and collecting the supernatant. Deidentified semen samples from patients with EDO (diagnosed by low seminal levels of fructose because fructose in semen originates from the seminal vesicles) were kindly provided by Olë Sorensen (Lund University, Sweden). Most infectivity experiments used seminal fluid rather than semen; the exception was the analysis of individual semen samples because there was not enough material to generate seminal fluid for the assays. Notably, the viral-enhancing activities of semen and seminal fluid are similar (Münch et al., 2007). Use of semen samples was conducted under guidelines approved by the Committee on Human Research at the University of California, San Francisco.

Depletion of Cationic and Anionic Components from Semen

SCX and SAX Dynabeads (Invitrogen, Carlsbad, CA) were used according to the manufacturer's protocol. Briefly, after washing and equilibration, Dynabeads were incubated with 20% pooled seminal fluid (diluted in PBS) for 1 hr at 4°C. A total of four rounds of depletion were carried out for each condition. For mock-depleted samples, 20% pooled seminal fluid samples were carried through the four rounds of 4°C incubations in the absence of added beads. Total protein content in the samples was determined by the bicinchoninic acid assay (Thermo Scientific, Waltham, MA).

ELISA

Immunolon II plates (Fisher, Waltham, MA) were coated for 12–18 hr at 4°C with 1% semen or the indicated concentration of antigen diluted in PBS. Next, wells were blocked for 2 hr at room temperature with 1% BSA diluted in PBS. Wells were then incubated with sera (diluted in PBS/1%/BSA) for 1 hr at room temperature. After washing, wells were incubated with horse radish peroxidase-conjugated secondary antibody (GE Healthcare, South San Francisco, CA) diluted in PBS/1% BSA. After a second wash, tetramethylbenzidine (TMB) substrate (Sigma-Aldrich, St. Louis MO) was added and the reaction was stopped with 2M H_2SO_4. OD at 450/650 nm was monitored using a spectrophotometer. Commercial monoclonal antibodies recognizing PSA (O14) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Commercial polyclonal antibodies recognizing PAP, lactoferrin, and albumin were purchased from Sigma-Aldrich. Amyloid-specific antibodies WO1 and WO2 were kindly provided by Ron Wetzel (University of Pittsburgh). Antibodies against PAP248–286, SEM1(49–107), and SEM2(49–107) were custom-produced by Pocono Rabbit Farms (Canadensis, PA).

Thioflavin T

125 μg/ml of agitated peptides were incubated with 5 μM Thioflavin T (Sigma-Aldrich), and the increase in fluorescence (excitation 440 nm, emission 482 nm) in triplicate wells was assayed with an Ls-5B luminescence spectrometer (Perkin-Elmer, Waltham, MA).

Electron Microscopy

Agitated peptides were adsorbed on glow-discharged carbon-coated grids and negatively stained with 2% uranyl acetate. For imaging of virions with

fibrils, envelope-defective HIV particles were centrifuged for 70 min at 14,000 rpm, and the pellet was resuspended in PBS and incubated at 4°C for 1 hr. Viral particles were then incubated with 500 μg/ml SEM1(49–107) fibrils for 10 min and fibrils were pelleted at 14,000 rpm for 10 min. Pellets were resuspended and adjusted to a peptide concentration of 5 mg/ml and prepared for electron microscopy. Samples were adsorbed on glow-discharged coated grids, fixed with 2.5% glutaraldehyde in phosphate buffer (pH 7.3, 0.2 M), and negatively stained with 0.5% uranyl acetate. All samples were imaged with a Zeiss EM10 transmission electron microscope (Zeiss, Oberkochen, Germany) at an accelerating voltage of 80 kV.

Fluorescence Microscopy

SEM1(49–107) fibrils (200 μg/ml) were added to the ProteoStat® Amyloid Plaque Detection Kit (Enzo Life Sciences, Plymouth Meeting, PA). Fibrils were then incubated 1:1 with MLV-gag-YFP viral particles and added, where indicated, to 50,000 TZMbl cells stained with CellTrace™ Violet Cell Proliferation Kit (Invitrogen). Samples were transferred onto μ-slides VI$^{0.4}$ (Ibidi, Munich, Germany) and imaged with a Zeiss LSM confocal microscope using LSM 710 Release version 5.5SP1 software.

Cell Culture

Primary CD4+ T cells were isolated from buffy coats by Ficoll-Hypaque density gradients, followed by negative selection with CD14+ microbeads and positive selection with CD4+ microbeads (Miltenyi, Bergisch Gladbach, Germany). The purified CD14-CD4+ cells were cultured in RPMI medium supplemented with 10% FBS, L-glutamine (2 mM), penicillin (50 U/ml), and streptomycin (50 μg/ml). 293T and TZM-bl cells were cultured in DMEM medium supplemented with 10% FBS, L-glutamine (2 mM), penicillin (50 U/ml), and streptomycin (50 μg/ml).

Viral Production

The CCR5-tropic 81A viruses used in TZM-bl infectivity assays were produced by Fugene-mediated transfection (Roche, Switzerland) of 293T cells with a proviral DNA expression plasmid. The CCR5-tropic NL4-3 92th014.12 viruses (Papkalla et al., 2002) used in TZM-bl infectivity assays were produced by transient calcium phosphate transfection of 293T cells with the proviral pBRHIV-1NL4-3_92Th014 expression plasmid. The BlaM-Vpr chimeric CCR5-tropic 81A and NL43 viruses used in the fusion assays were produced by Fugene-mediated transfection of 293T cells with proviral DNA expression plasmids as previously described (Cavrois et al., 2002). Two days after transfection, supernatants were clarified by sedimentation and titered for p24 content by anti-p24^{Gag} ELISA (Perkin-Elmer).

Virion Fusion Assay

Virion fusion was performed similar to previously described methods (Cavrois et al., 2002). HIV-1 virions (200 ng/ml) were pretreated with SEM or SEVI fibrils (31.25 μg/ml) for 1 hr at 37°C and then added to 5 × 10^5 CD14-CD4+ target cells. To examine the effect of heparin on the viral-enhancing activity of the SEM fibrils, target cells, rather than virions, were pretreated because heparin has direct inhibitory effects on HIV (Roan et al., 2009). For target cell pretreatment, 5 × 10^5 CD14-CD4+ cells were incubated with the indicated SEM fibrils (31.25 μg/ml) in the absence or presence of heparin (31.25 μg/ml, Sigma-Aldrich) for 1 hr. After pretreatment, cells were washed three times with medium and added to virions. Viral fusion was allowed to proceed for 4 hr at 37°C, after which cells were loaded overnight with CCF2. Cells were stained with surface antibodies against CD3 and CD4, fixed with 2% paraformaldehyde, and then analyzed on a BD LSRII. Flow cytometric data were processed with FlowJo software (Treestar, Ashland, OR).

TZM-bl Infectivity Assays

Infectivity assays were essentially performed as described (Kim et al., 2010; Roan et al., 2009). HIV-1 from the supernatant of transfected 293T cells was diluted to 0.1 μg/ml p24 and pretreated for 5 min with peptide or semen/seminal fluid at the indicated concentrations. Pretreated virions (20 μl) were added to TZM-bl cells (10^4 /well in 96-well flat-bottom plates) in 180 μl medium (for peptide treatment) or 280 μl medium (for semen/seminal fluid treatment). To minimize toxic effects mediated by prolonged exposure of semen/ seminal fluid to target cells, the medium was replaced after 2 hr, and infection was assayed 2–3 days later by monitoring β-galactosidase activity using the Gal-Screen kit (Life Technologies, Carlsbad, CA). Background signals obtained from uninfected cells were subtracted from values obtained with infected cells.

Western Blotting

The equivalent of 0.2–1 μl of seminal fluid was loaded onto 12.5% Criterion Tris-HCl polyacrylamide gels (Bio-Rad, Hercules, CA). Proteins were transferred onto nitrocellulose iBlot membranes (Invitrogen) and blocked with PBS in the presence of 5% milk and 0.1% Tween-20. Commercial antibodies against SEM1/2 and PSA (sc33819 and sc80304) were purchased from Santa Cruz Biotechnology, commercial antibodies against albumin and PAP (A0433 and P5664) were purchased from Sigma, and custom antibodies against PAP248–286, SEM1(49–107), and SEM2(49–107) were custom-produced by Pocono Rabbit Farms. Primary antibodies were used at a dilution of 1:1,000–1:40,000, and HRP-conjugated secondary antibodies were used at a final dilution of 1:5,000. Blots were developed using the Western Lightning ECL kit (Perkin-Elmer).

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, one table, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.chom.2011.10.010.

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REFERENCES

Ariel, A., Fredman, G., Sun, Y.P., Kantarci, A., Van Dyke, T.E., Luster, A.D., and Serhan, C.N. (2006). Apoptotic neutrophils and T cells sequester chemokines during immune response resolution through modulation of CCR5 expression. Nat. Immunol. *7*, 1209–1216.

Balandya, E., Sheth, S., Sanders, K., Wieland-Alter, W., and Lahey, T. (2010). Semen protects CD4+ target cells from HIV infection but promotes the preferential transmission of R5 tropic HIV. J. Immunol. *185*, 7596–7604.

Bjartell, A., Malm, J., Moller, C., Gunnarsson, M., Lundwall, A., and Lilja, H. (1996). Distribution and tissue expression of semenogelin I and II in man as demonstrated by in situ hybridization and immunocytochemistry. J. Androl. *17*, 17–26.

Bouhlal, H., Chomont, N., Haeffner-Cavaillon, N., Kazatchkine, M.D., Belec, L., and Hocini, H. (2002). Opsonization of HIV-1 by semen complement enhances infection of human epithelial cells. J. Immunol. *169*, 3301–3306.

Bursell, S. (1942). Beitrag zur Kenntnis der Para-amyloidose im urngenitalen System unter besonderer Berücksichtigung der sog, senilen Amyloidose in den Samenbläschen und ihres Verhältnisses zum Samenbläschenpigment. Upsala Läkaref Förh *47*, 313–331.

Cavrois, M., De Noronha, C., and Greene, W.C. (2002). A sensitive and specific enzyme-based assay detecting HIV-1 virion fusion in primary T lymphocytes. Nat. Biotechnol. *20*, 1151–1154.

Ceballos, A., Remes Lenicov, F., Sabatté, J., Rodríguez Rodrígues, C., Cabrini, M., Jancic, C., Raiden, S., Donaldson, M., Agustín Pasqualini, R., Jr., Marin-Briggiler, C., et al. (2009). Spermatozoa capture HIV-1 through heparan sulfate and efficiently transmit the virus to dendritic cells. J. Exp. Med. *206*, 2717–2733.

de Lamirande, E. (2007). Semenogelin, the main protein of the human semen coagulum, regulates sperm function. Semin. Thromb. Hemost. *33*, 60–68.

de Lamirande, E., Yoshida, K., Yoshiike, T.M., Iwamoto, T., and Gagnon, C. (2001). Semenogelin, the main protein of semen coagulum, inhibits human sperm capacitation by interfering with the superoxide anion generated during this process. J. Androl. *22*, 672–679.

Doncel, G.F. (2006). Exploiting common targets in human fertilization and HIV infection: development of novel contraceptive microbicides. Hum. Reprod. Update *12*, 103–117.

Edström, A.M., Malm, J., Frohm, B., Martellini, J.A., Giwercman, A., Mörgelin, M., Cole, A.M., and Sørensen, O.E. (2008). The major bactericidal activity of human seminal plasma is zinc-dependent and derived from fragmentation of the semenogelins. J. Immunol. *181*, 3413–3421.

Hauber, I., Hohenberg, H., Holstermann, B., Hunstein, W., and Hauber, J. (2009). The main green tea polyphenol epigallocatechin-3-gallate counteracts semen-mediated enhancement of HIV infection. Proc. Natl. Acad. Sci. USA *106*, 9033–9038.

Herr, J.C., Summers, T.A., McGee, R.S., Sutherland, W.M., Sigman, M., and Evans, R.J. (1986). Characterization of a monoclonal antibody to a conserved epitope on human seminal vesicle-specific peptides: a novel probe/marker system for semen identification. Biol. Reprod. *35*, 773–784.

Iwamoto, T., and Gagnon, C. (1988). A human seminal plasma protein blocks the motility of human spermatozoa. J. Urol. *140*, 1045–1048.

Kayed, R., Head, E., Sarsoza, F., Saing, T., Cotman, C.W., Necula, M., Margol, L., Wu, J., Breydo, L., Thompson, J.L., et al. (2007). Fibril specific, conformation dependent antibodies recognize a generic epitope common to amyloid fibrils and fibrillar oligomers that is absent in prefibrillar oligomers. Mol. Neurodegener. *2*, 18.

Kim, K.A., Yolamanova, M., Zirafi, O., Roan, N.R., Staendker, L., Forssmann, W.G., Burgener, A., Dejucq-Rainsford, N., Hahn, B.H., Shaw, G.M., et al. (2010). Semen-mediated enhancement of HIV infection is donor-dependent and correlates with the levels of SEVI. Retrovirology *7*, 55.

Koistinen, H., Soini, T., Leinonen, J., Hyden-Granskog, C., Salo, J., Halttunen, M., Stenman, U.H., Seppälä, M., and Koistinen, R. (2002). Monoclonal antibodies, immunofluorometric assay, and detection of human semenogelin in male reproductive tract: no association with in vitro fertilizing capacity of sperm. Biol. Reprod. *66*, 624–628.

Linke, R.P., Joswig, R., Murphy, C.L., Wang, S., Zhou, H., Gross, U., Rocken, C., Westermark, P., Weiss, D.T., and Solomon, A. (2005). Senile seminal vesicle amyloid is derived from semenogelin I. J. Lab. Clin. Med. *145*, 187–193.

Liu, Z., and Foote, R.H. (1998). Bull sperm motility and membrane integrity in media varying in osmolality. J. Dairy Sci. *81*, 1868–1873.

Mandal, A., and Bhattacharyya, A.K. (1995). Sperm hyaluronidase activation by purified predominant and major basic human seminal coagulum proteins. Hum. Reprod. *10*, 1745–1750.

Martellini, J.A., Cole, A.L., Venkataraman, N., Quinn, G.A., Svoboda, P., Gangrade, B.K., Pohl, J., Sørensen, O.E., and Cole, A.M. (2009). Cationic polypeptides contribute to the anti-HIV-1 activity of human seminal plasma. FASEB J. *23*, 3609–3618.

Miller, C.J., Marthas, M., Torten, J., Alexander, N.J., Moore, J.P., Doncel, G.F., and Hendrickx, A.G. (1994). Intravaginal inoculation of rhesus macaques with cell-free simian immunodeficiency virus results in persistent or transient viremia. J. Virol. *68*, 6391–6400.

Münch, J., Rücker, E., Ständker, L., Adermann, K., Goffinet, C., Schindler, M., Wildum, S., Chinnadurai, R., Rajan, D., Specht, A., et al. (2007). Semen-derived amyloid fibrils drastically enhance HIV infection. Cell *131*, 1059–1071.

Neildez, O., Le Grand, R., Chéret, A., Caufour, P., Vaslin, B., Matheux, F., Théodoro, F., Roques, P., and Dormont, D. (1998). Variation in virological parameters and antibody responses in macaques after atraumatic vaginal exposure to a pathogenic primary isolate of SIVmac251. Res. Virol. *149*, 53–68.

O'Nuallain, B., and Wetzel, R. (2002). Conformational Abs recognizing a generic amyloid fibril epitope. Proc. Natl. Acad. Sci. USA *99*, 1485–1490.

Olsen, J.S., Brown, C., Capule, C.C., Rubinshtein, M., Doran, T.M., Srivastava, R.K., Feng, C., Nilsson, B.L., Yang, J., and Dewhurst, S. (2010). Amyloid-binding small molecules efficiently block SEVI (semen-derived enhancer of virus infection)- and semen-mediated enhancement of HIV-1 infection. J. Biol. Chem. *285*, 35488–35496.

Papkalla, A., Münch, J., Otto, C., and Kirchhoff, F. (2002). Nef enhances human immunodeficiency virus type 1 infectivity and replication independently of viral coreceptor tropism. J. Virol. *76*, 8455–8459.

Pitkänen, P., Westermark, P., Cornwell, G.G., III, and Murdoch, W. (1983). Amyloid of the seminal vesicles. A distinctive and common localized form of senile amyloidosis. Am. J. Pathol. *110*, 64–69.

Pryor, J.P., and Hendry, W.F. (1991). Ejaculatory duct obstruction in subfertile males: analysis of 87 patients. Fertil. Steril. *56*, 725–730.

Roan, N.R., Münch, J., Arhel, N., Mothes, W., Neidleman, J., Kobayashi, A., Smith-McCune, K., Kirchhoff, F., and Greene, W.C. (2009). The cationic properties of SEVI underlie its ability to enhance human immunodeficiency virus infection. J. Virol. *83*, 73–80.

Roan, N.R., Sowinski, S., Münch, J., Kirchhoff, F., and Greene, W.C. (2010). Aminoquinoline surfen inhibits the action of SEVI (semen-derived enhancer of viral infection). J. Biol. Chem. *285*, 1861–1869.

Robert, M., and Gagnon, C. (1995). Sperm motility inhibitor from human seminal plasma: association with semen coagulum. Hum. Reprod. *10*, 2192–2197.

Robert, M., and Gagnon, C. (1996). Purification and characterization of the active precursor of a human sperm motility inhibitor secreted by the seminal vesicles: identity with semenogelin. Biol. Reprod. *55*, 813–821.

Robert, M., and Gagnon, C. (1999). Semenogelin I: a coagulum forming, multifunctional seminal vesicle protein. Cell. Mol. Life Sci. *55*, 944–960.

Robert, M., Gibbs, B.F., Jacobson, E., and Gagnon, C. (1997). Characterization of prostate-specific antigen proteolytic activity on its major physiological substrate, the sperm motility inhibitor precursor/semenogelin I. Biochemistry *36*, 3811–3819.

Rönnberg, L., Vihko, P., Sajanti, E., and Vihko, R. (1981). Clomiphene citrate administration to normogonadotropic subfertile men: blood hormone changes and activation of acid phosphatase in seminal fluid. Int. J. Androl. *4*, 372–378.

Sabatté, J., Ceballos, A., Raiden, S., Vermeulen, M., Nahmod, K., Maggini, J., Salamone, G., Salomón, H., Amigorena, S., and Geffner, J. (2007). Human seminal plasma abrogates the capture and transmission of human immunodeficiency virus type 1 to CD4+ T cells mediated by DC-SIGN. J. Virol. *81*, 13723–13734.

Toyama, B.H., and Weissman, J.S. (2011). Amyloid structure: conformational diversity and consequences. Annu. Rev. Biochem. *80*, 557–585.

Yoshida, K., Yamasaki, T., Yoshiike, M., Takano, S., Sato, I., and Iwamoto, T. (2003). Quantification of seminal plasma motility inhibitor/semenogelin in human seminal plasma. J. Androl. *24*, 878–884.

Zalensky, A.O., Yau, P., Breneman, J.W., and Bradbury, E.M. (1993). The abundant 19-kilodalton protein associated with human sperm nuclei that is related to seminal plasma alpha-inhibins. Mol. Reprod. Dev. *36*, 164–173.

Supporting Data

Figure Legends

Figure 1. Superfibronectin and seminal fibronectin enhance HIV-1 infection. (A) Cells were treated with the indicated concentration of fibronectin, superfibronectin, or BSA, washed, and then coated with CCR5-tropic 81A HIV-1. TZMbl cells were added, and cells were assayed for Tat-inducible β-galactosidase activity 3 days later. The numbers above the bars indicate n-fold infectivity enhancement relative to infection measured in the absence of peptide. (B) Seminal fluid was measured by ELISA for levels of fibronectin following immunodepletion with anti-fibronectin, anti-lactoferrin, and anti-albumin. (C) Samples in (B) were tested in an infectivity assay similar to that described in (A), except that diluted seminal fluid instead of purified proteins were used. Abbreviations: RLU/s: relative light units / second, FN: fibronectin, Lfn: Lactoferrin, Alb: Albumin. *$p<0.05$ by the student's t test.

Figure 2. Association of SEM amyloid levels with increased semen/blood HIV viral load ratio. Significant correlation was observed between the semen-to-blood HIV viral load ratio and semen SEM2(49-107) level.

Figure 3. SEM amyloids and seminal fluid induce IL6 production. (A) PMA, LPS, SEM fibrils, or a monomeric SEM peptide was added to CD4+ PBLs, and supernatants were analyzed 24h later for the levels of IL6. (B) CD4+ PBLs were exposed to the indicated concentration of seminal fluid (SF) and IL6 production was assessed by ELISA. (C) IL6 production was assessed in primary endometrial epithelial cells following co-culture with 1% SF or media as a control.

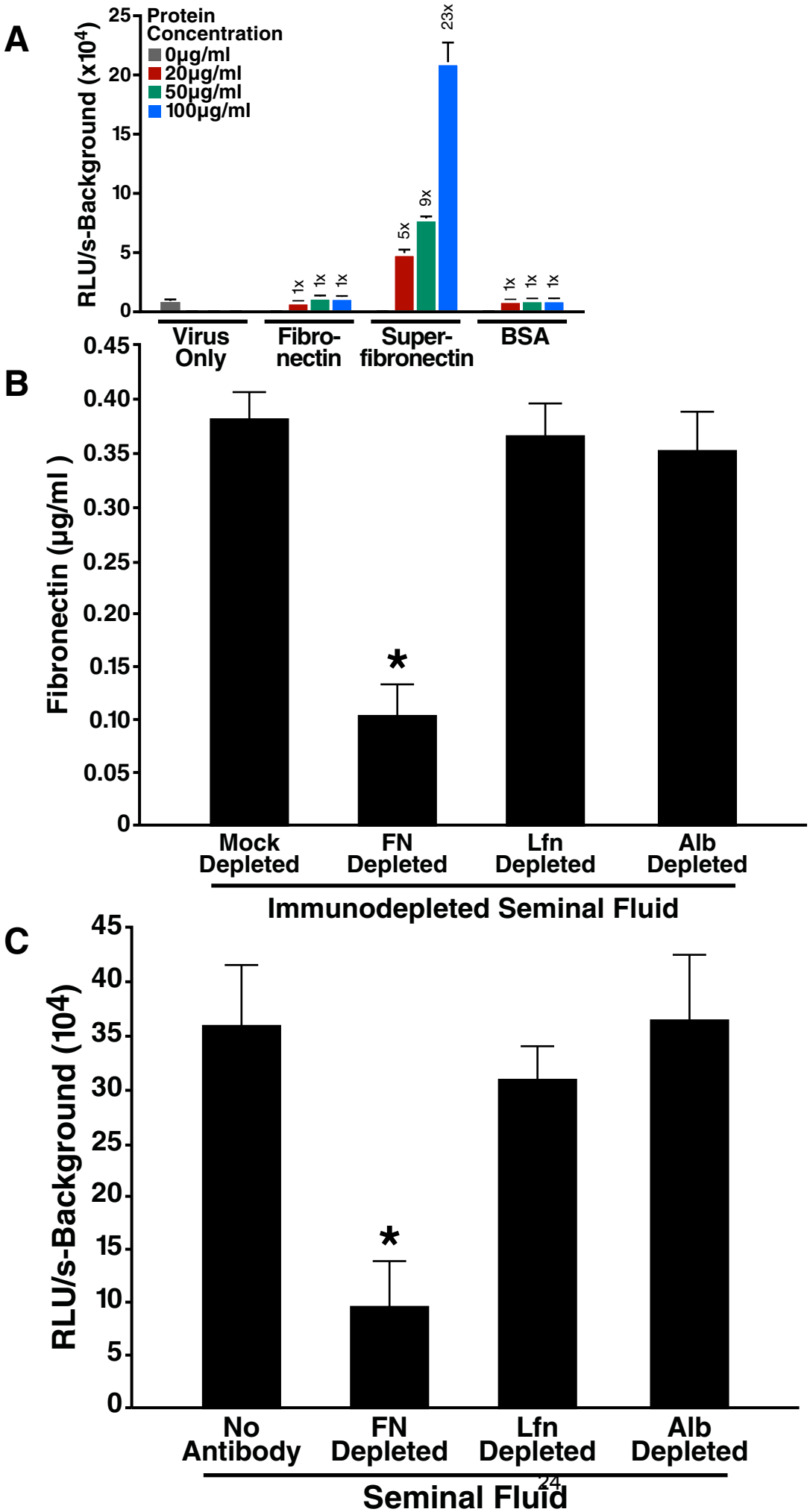
Figure 4. Seminal fluid (SF) decreases epithelial monolayer integrity. (A) Primary vaginal epithelial monolayers were treated as indicated, and transepithelial resistance (TER), a measure of barrier integrity, was measured after 72 hours. (B) Viability of the samples in (A) was measured by quantifying relative ATP levels. (C) The tight junction proteins ZO-1 and occludin were quantified following treatment with SF. (D) Fluorescence microscopy demonstrating the decrease in barrier integrity (as assessed by ZO-1 staining) following treatment with SF.

Figure 5. (A) The chemical structures of surfen and its structural analogs 4-aminoquinaldine, 1,3-di-6-quinolylurea, and scarlet acid. (B) Comparing the inhibitory activity of surfen and 4-aminoquinaldine, 1,3-di-6-quinolylurea, or scarlet acid on semen-mediated enhancement of HIV infection. 81A virions were pretreated for 5 min with the indicated concentration of semen in the presence of 0, 10, 50, or 100 μm surfen or its analogs. The samples were then diluted 15-fold (to final inhibitor concentrations of 0, 0.67, 3.3, or 6.7 μm as indicated) and added to TZM-bl cells. Medium was replaced after 2 h, and cells were assayed for Tat-inducible β-galactosidase activity 3 days later. RLU/s, relative light units/s. Shown are average values (± S.D.) of triplicate measurements.

Figure 6. List of hits from the pilot screen of semen viral enhancer inhibitors.

Figure 7. SEVI enhances HIV-1 transmission *in vivo* in MISTRG humanized mice. Mice were either mock-infected, infected intravaginally with HIV-1 (1.6 ng p24), or infected intravaginally with HIV-1 (1.6 ng p24) in the presence of SEVI (100 μg/ml). Three days later, spleens were harvested and stained for intracellular p24. Shown are the % of CD4+ T cell containing intracellular p24 (A) or the mean fluorescence of p24 in CD4+ T cells (B).

Figure 1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Protein Concentration
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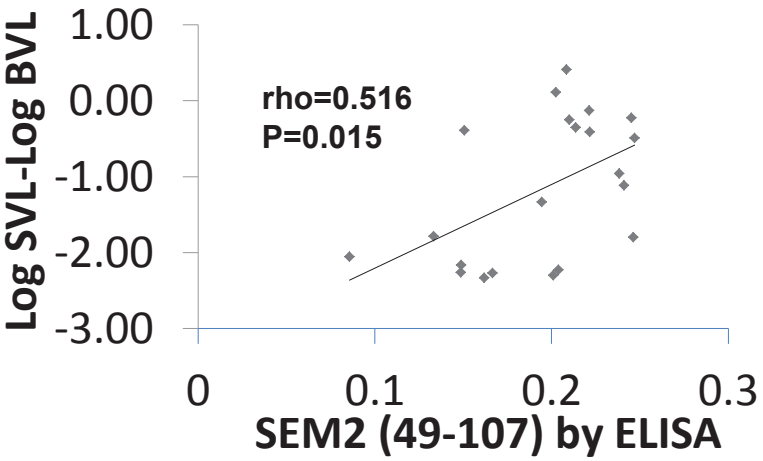
20µg/ml
50µg/ml
100µg/ml
RLU/s-Background (x10^4)
25
20
15
10
5
0
1x
1x
1x
5x
9x
23x
1x
1x
1x
Virus Only
Fibro-nectin
Super-fibronectin
BSA
B
Fibronectin (µg/ml)
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
*
Mock Depleted
FN Depleted
Lfn Depleted
Alb Depleted
Immunodepleted Seminal Fluid
C
RLU/s-Background (10^4)
45
40
35
30
25
20
15
10
5
0
*
No Antibody
FN Depleted
Lfn Depleted
Alb Depleted
Seminal Fluid

Figure 2


Log SVL-Log BVL
1.00
0.00
-1.00
-2.00
-3.00
rho=0.516
P=0.015
0
0.1
0.2
0.3
SEM2 (49-107) by ELISA

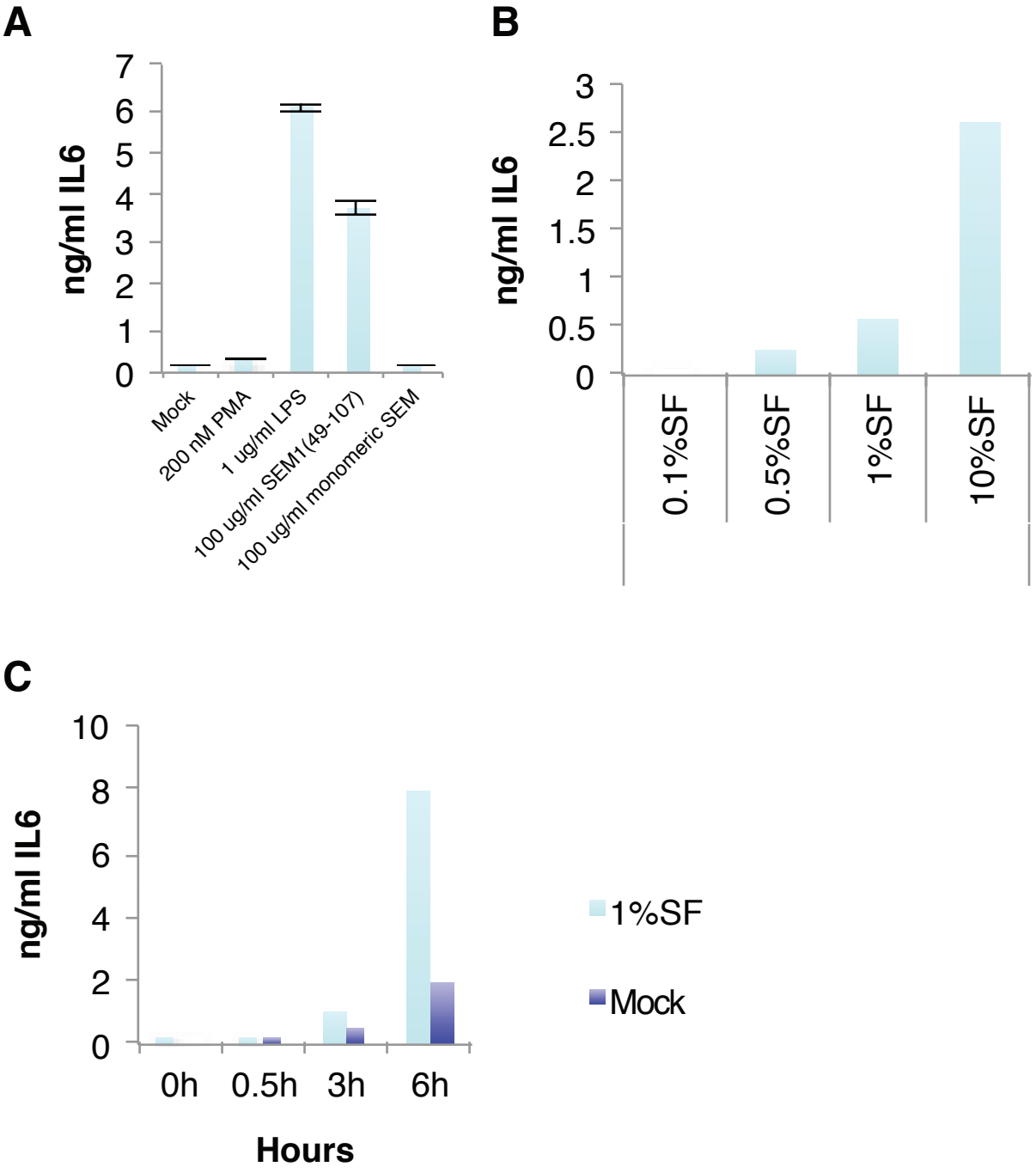
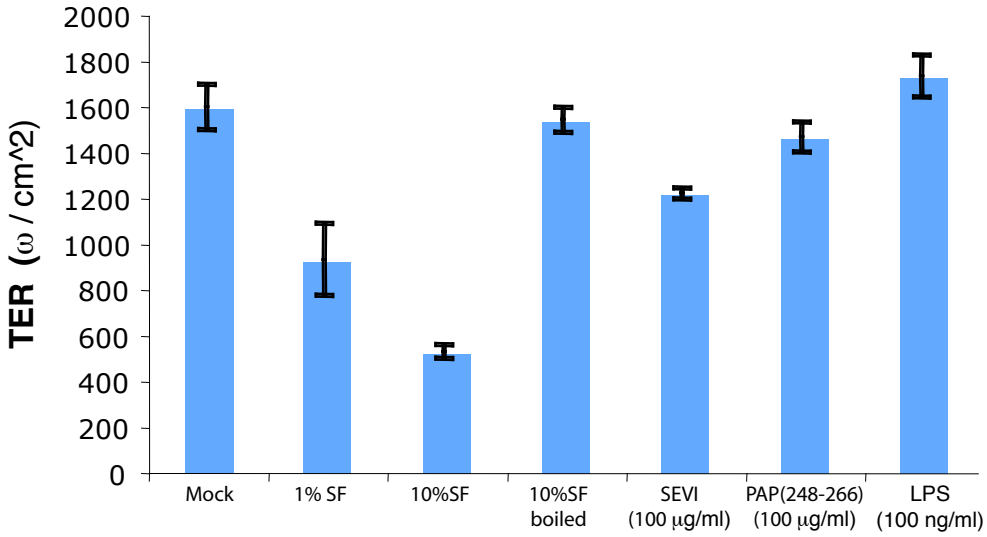

A
ng/ml IL6
7
6
5
4
3
2
1
0
Mock
200 nM PMA
1 ug/ml LPS
100 ug/ml SEM1(49-107)
100 ug/ml monomeric SEM
B
ng/ml IL6
3
2.5
2
1.5
1
0.5
0
0.1%SF
0.5%SF
1%SF
10%SF
C
ng/ml IL6
10
8
6
4
2
0
0h
0.5h
3h
6h
Hours
1%SF
Mock

Figure 4

A



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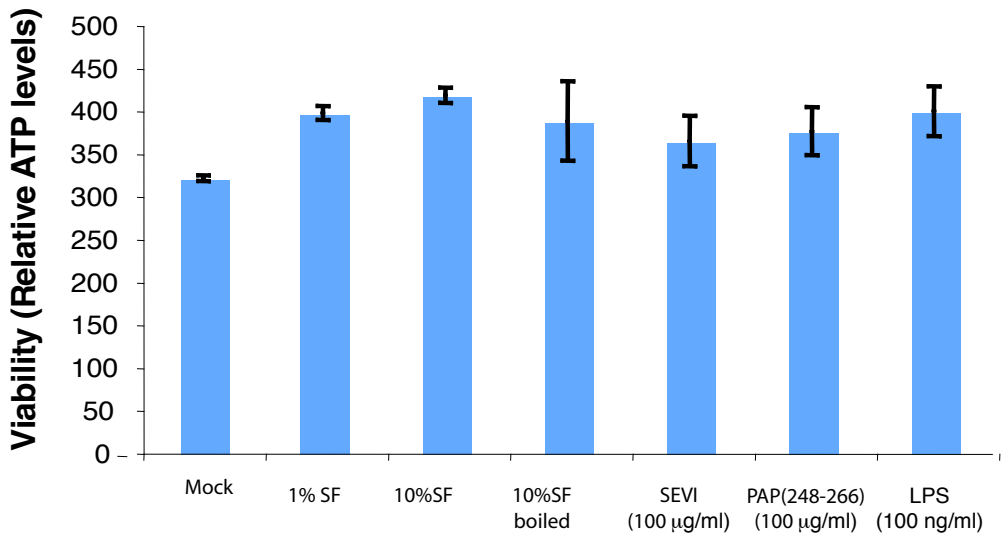

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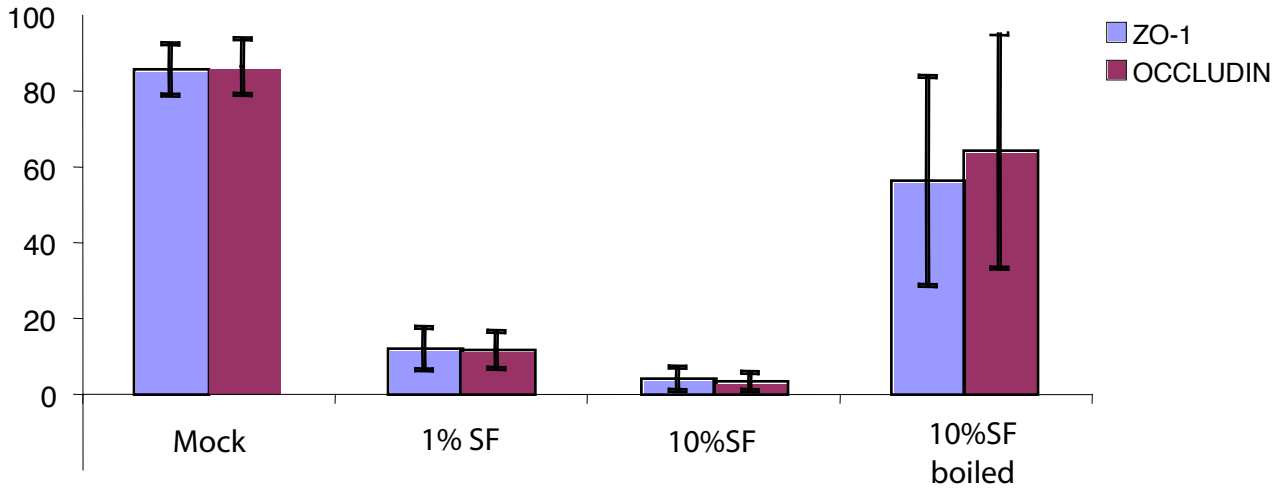

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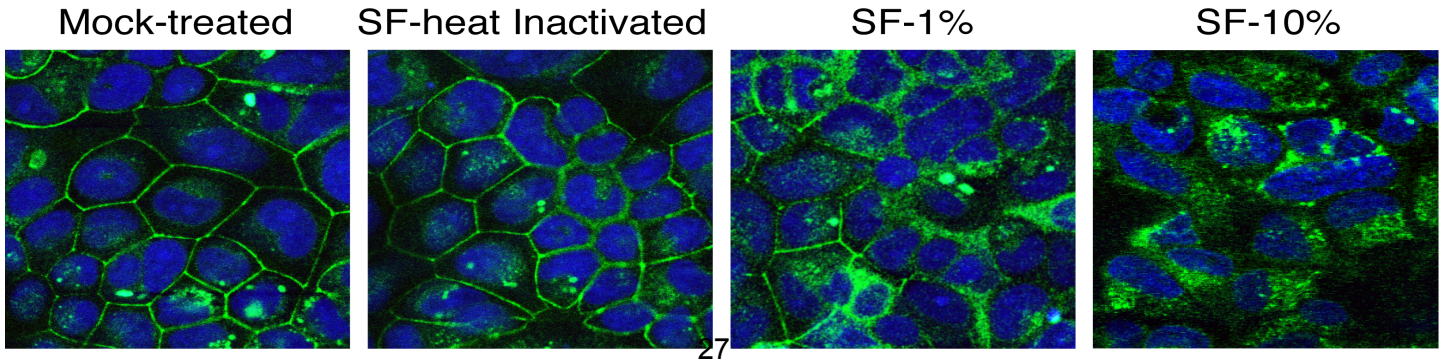
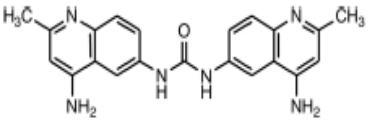


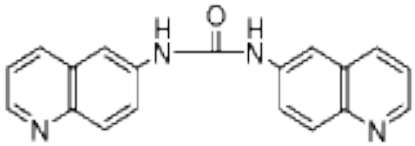
Figure 5

A

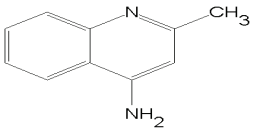
Surfen



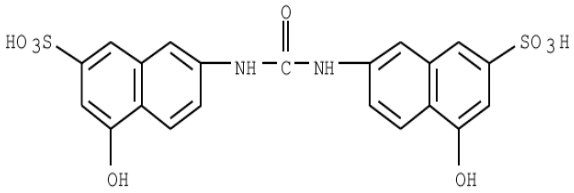
1,3-di-6-quinolylurea



4-aminoquinaldine



Scarlet acid



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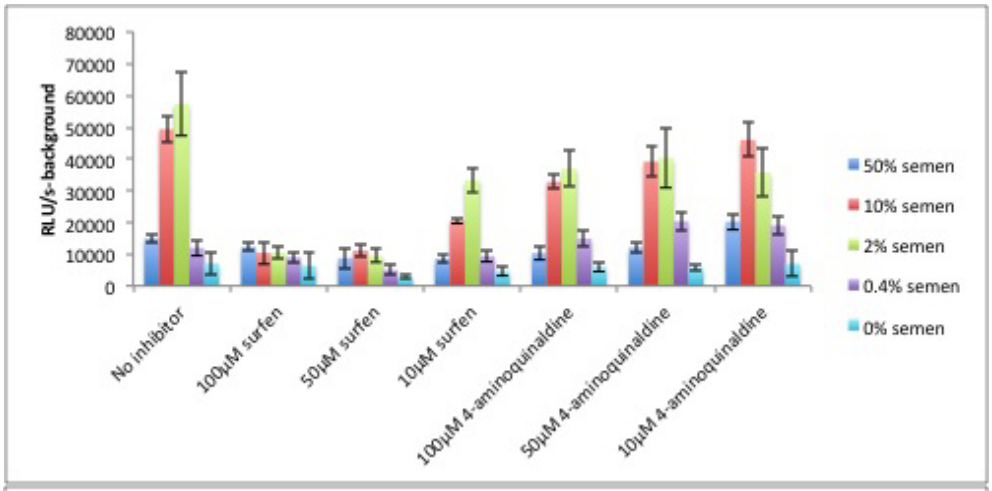
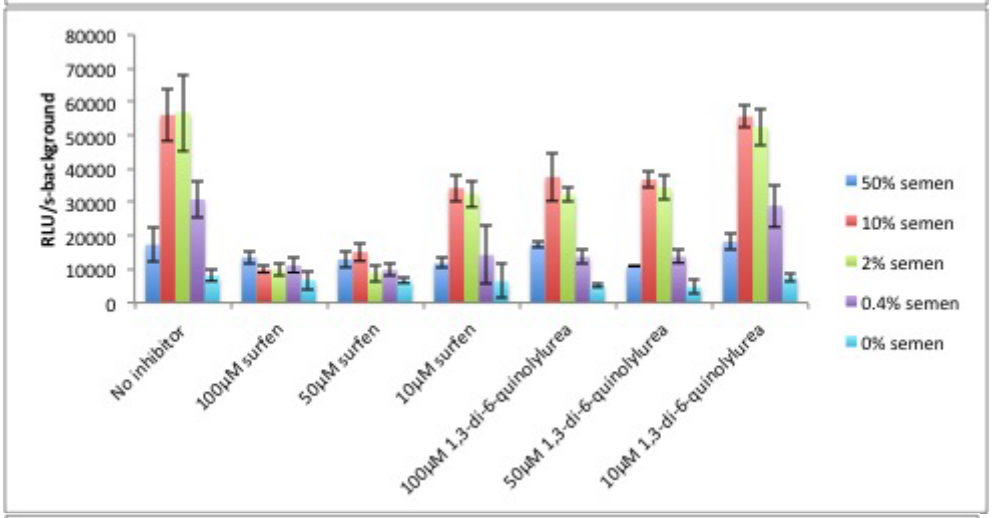
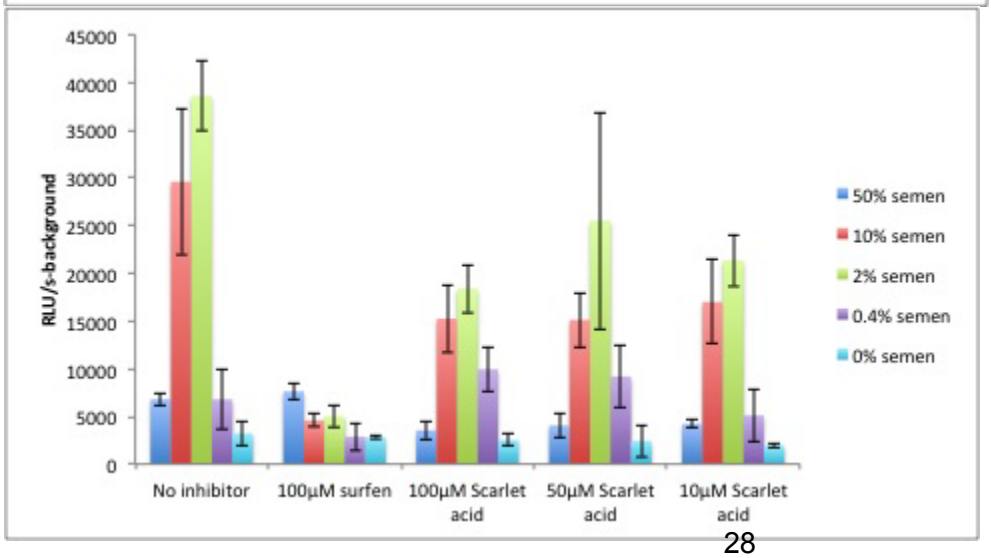






aloin

7-deshydroxypyrogallin-4-carboxylic acid

Sanguinarine chloride

quercetin

levodopa

emodic acid

retusoquinone

pyrogallin

alizarin

dehydrodeoxysappanone b dimethyl ether

baicalein

5,7-dihydroxyisoflavone

haematoxylin

carminic acid

quinalizarin

resveratrol

2',2'-bisepigallocatechin digallate

theaflavin digallate

pararosaniline pamoate

isoliquiritigenin

methyl 7-deshydroxypyrogallin-4-carboxylate

2',2'-bisepigallocatechin monogallate

purpurogallin

gossypol

iriginol hexaaceatate

irigenol

merbromin

phenazopyridine hydrochloride

prazosin hydrochloride

pyrvinium pamoate

sulfasalazine

rhamnetin

fisetin

hematein

morin

centaurein

diosmetin

hieracin

3,5-Dinitrocatechol

homidium bromide

menadione

dantrolene sodium

myricetin

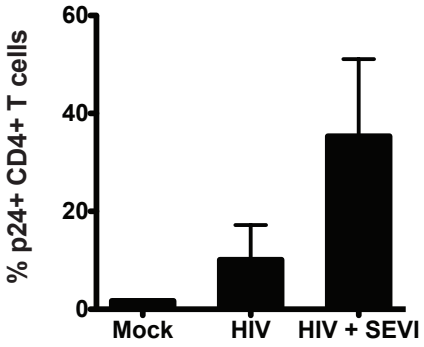
arthonioic acid

mechlorethamine

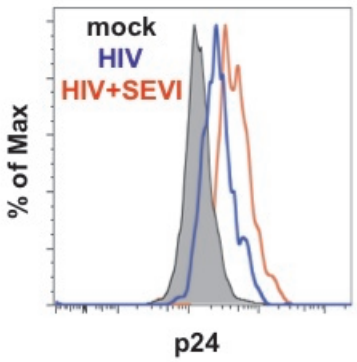
5-Nitro-2-(3-phenylpropylamino) benzoic acid

Figure 7

A


% p24+ CD4+ T cells
60
40
20
0
Mock
HIV
HIV + SEVI

B


mock
HIV
HIV+SEVI
% of Max
p24